THE ROLE OF ALPHA INTERFERONS IN THE RECOGNITION OF PREGNANCY IN CATTLE

BY

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1990

To our Lord who directed me with his hands
and to my husband, John, whose faith and encouragement
made this achievement possible

ACKNOWLEDGMENTS

The author gratefully thanks the chairman and co-chairman of her advisory committee, Drs. Peter J. Hansen and William W. Thatcher, for their enthusiastic participation and helpful guidance throughout her doctoral program. She also acknowledges the assistance received by the remaining members of her advisory committee, Drs. Fuller W. Bazer, William C. Buhi and Marteen Drost.

I am indebted to Ms. Marie V. Leslie, Mr. Jesse Johnson and Mr. Frank Michel for their technical assistance, to Mr. Dale Hissem and Mr. Austin Green for their cheerful help in handling animals, to Mr. Larry Eubanks for his assistance in obtaining tissues and Mrs. Mary-Ellen Hissem for her joyful personality and skill in typing.

The author also wishes to acknowledge the help and friendship of fellow graduate students and postdoctoral trainees.

I would like to express my gratitude to Dr. Charles Wilcox for his statistical advice and the happy moments spent in his office talking about Montreal.

Special thanks go to Dr. Richard B. Miller at the University of Guelph for his encouragement and support in the writing of her dissertation, to Dr. Keith J. Betteridge to allow the use of his laser printer and to her sister, Louise, for editorial assistance.

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ABBREVIATIONS

1D SDS-PAGE	one dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis
2D SDS-PAGE	two dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis
bCSP	bovine conceptus secretory proteins
bTP-1	bovine trophoblast protein-1
BoIFN	bovine interferon alpha, class I, type 1
BSA	bovine serum albumin
CL	corpus luteum
CV	coefficient of variation
DATD	N,N'-diallyltartardiamide
D-PBS	Dulbecco's phosphate buffered saline
E_2	estradiol-17β
EDTA	sodium ethylenediamine tetraacetate
ELISA	enzyme-linked immunosorbent assay
EPSI	endometrial prostaglandin synthesis inhibitor
hCG	human chorionic gonadotropin
HPLC	high performance liquid chromatography
HuIFN	human interferon alpha, class I
IFN	interferon class α molecules
IM	intramuscular injection
IU	intrauterine infusion
mRNA	messenger ribonucleic acid
MEM	modified Eagle's minimum essential medium
NP-40	nonidet P-40
oCSP	ovine conceptus secretory proteins
oTP-1	ovine trophoblast protein-1
PGE_2	prostaglandin E_2
PGF	prostaglandin F
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGFM	13,14-dihydro-15-keto-$PGF_{2\alpha}$
PMSF	phenylmethylsulfonyl fluoride
rBoIFN	recombinant bovine interferon alpha, class I, type 1
rHuIFN	recombinant human interferon alpha, class I
RIA	radioimmunoassay
SA	specific activity
SDS	sodium dodecyl sulfate
s.e.m.	standard error of the means
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TP-1	trophoblast protein-1

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

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May 1990

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Major Department: Animal Science

The bovine conceptus must signal its presence to the maternal system to block regression of the corpus luteum (CL) and the onset of a new estrous cycle. One of the primary signals is a complex of related proteins known as bovine trophoblast protein-1 (bTP-1) which shares high amino acid sequence homology with interferon alpha molecules. Bovine TP-1 is hypothesized to suppress endometrial synthesis of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Present results indicated that Day 17-18 bovine conceptuses secrete nine bTP-1 isomers and an additional four that are in the bTP-1 region (Mr classes: 26, 23, 21 kDa). Secretion of bTP-1 by cultured conceptuses occurred at a constant rate for 3 days and then declined on the fourth day of culture. The bTP-1 complex was purified using ultrafiltration followed by HPLC chromatography. Purified bTP-1 contained nine isomers and displayed antiviral activity. Immunological studies confirmed the high degree of homology between bTP-

1, oTP-1, recombinant bovine interferon alpha$_I$ (rBoIFN) and human interferon alpha$_I$. Antiserum raised against the 152-172 amino acid bTP-1 fragment was effective in detecting bTP-1 by ELISA, Western blotting and immunocytochemistry. None of the three individual bTP-1 peptides (100-113, 131-140 and 152-172) displayed antiviral activity.

Intramuscular and intrauterine administration of rBoIFN extended interestrous interval and CL lifespan. Oxytocin-induced PGF metabolite (PGFM) was reduced by rBoIFN treatment. Administration of rBoIFN also was pyrogenic and caused an acute decrease in plasma progesterone concentrations. Number and size of follicles on Day 17 and estradiol concentrations on Days 14 to 17 were not affected by rBoIFN. In three out of four in vitro experiments, rBoIFN did not affect the release of PGF by endometrial explants from Day 17 cyclic cows but, secretion rate of PGE_2 was increased. In the other experiment, secretion rates of PGF and PGE_2 were diminished by rBoIFN, bTP-1 and ovine TP-1.

In conclusion, the bTP-1 complex contains at least 9 to 13 isomers and displays antiviral activity and immunological cross-reactivity with other alpha interferons. Results from in vivo experiments indicate that rBoIFN can extend luteal lifespan by blocking PGFM release. However, effects on body temperature and progesterone must be studied further before utilizing rBoIFN as a potential fertility-enhancing drug in cattle.

CHAPTER 1
INTRODUCTION

In order to maximize the probability of the establishment of a successful pregnancy, the bovine species has evolved a polyestrous form of reproductive behavior, wherein rhythmic cycles of hormonal secretion lead to the ovulation of mature oocytes every 21 days. During the luteal phase of the estrous cycle, ovarian secretion of progesterone prepares a suitable uterine environment for the survival of a developing embryo. If an embryo is not present or is not developing normally, a uterine luteolysin ($PGF_{2\alpha}$) causes destruction of the corpus luteum (CL). Ovarian secretion of progesterone is essential for the support of pregnancy in cattle until at least 200 days of gestation and consequently the conceptus acts on the endometrium around Day 15 of the cycle to block $PGF_{2\alpha}$ release and ensure conceptus survival. The events leading to the establishment and maintenance of pregnancy are not always successful, however. Approximately 30% of bred cows lose their embryos before Day 30 of gestation. Economic gain will be realized by both beef and dairy cattle industries if methods are developed to reduce this wastage of embryos.

The research for this dissertation was concerned with the characterization of in vitro secretion of a conceptus antiluteolysin known as bTP-1, the development of an improved purification scheme for bTP-1, and an evaluation of the relationship between bTP-1 and other interferon

molecules, with the eventual possibility of using these interferons to regulate fertility.

CHAPTER 2
REVIEW OF LITERATURE

Introduction

The bovine estrous cycle and the establishment and maintenance of pregnancy are areas that have stimulated much research in the past 20 years. This literature review will cover our present understanding of these topics, especially in cattle, with complementary comparisons with other species. Special attention will be paid to folliculogenesis and development of the CL, luteolytic mechanisms involved in cyclic regression of the CL, a description of the biochemical dialogue between the mother and the early developing conceptus and finally, a description of the artificial means of prolonging luteal lifespan.

The Estrous Cycle in Cattle

Cows that have reached puberty exhibit a behavioral estrus approximately every 21 days until a pregnancy has been successfully established. This cyclicity of behavioral estrus ensures that male-derived gametes will be present only around the time of ovulation. The period which begins with sexual receptivity (estrus) and ends with the subsequent period of sexual receptivity is defined as the estrous cycle. The length of the estrous cycle is 21 days (3.7 days, s.d.) for cows and 20 days (2.3 days, s.d.) for heifers

(Robinson, 1977), with a lower variation within animals than between animals (Wishart, 1972). It is common to denote the first day of estrus as Day 0 of the cycle and to divide the estrous cycle into four different stages: estrus, metestrus, diestrus and proestrus. Estrus is the period characterized by sexual desire wherein the female allows herself to be mounted (Blockey, 1980; Esslemont et al., 1980). Estrus lasts approximately 18 h, and ovulation occurs 10-12 h after the end of estrus (Hansel and Echternkamp, 1972; Wishart, 1972). Metestrus is a period of 3-5 days following estrus during which there is development of an early corpus luteum called the corpus hemorrhagicum. Metestrus is then followed by a 10-14 day period of diestrus which is characterized by a functional corpus luteum (CL). The CL regresses during a 2-3 day period associated with preovulatory follicular growth. This 2-3 day period, preceding the next estrus, corresponds to proestrus.

During the estrous cycle, these arise prominent physiological and morphological events associated with the ovarian cortex. They include follicular development, atresia and ovulation, as well as development, maintenance and regression of the CL.

Folliculogenesis

Stages of follicular growth. As the germ cells of the developing bovine conceptus enter meiosis, epithelial cells surround primary oocytes to form primordial follicles (Byskov and Lintern-Moore, 1973). The primordial follicle consists of an oocyte enclosed in a single layer of squamous cells which are destined to become the granulosa cell layer (see Peters, 1978). These resulting units form a pool of small non-growing follicles at birth. Under an unknown signal, the granulosa cells proliferate. This is followed by an organization of theca cells external to the basement membrane. Follicles,

containing a single layer of cuboidal granulosa cells and a primordial oocyte, secrete a glycoprotein structure around the oocyte known as the zona pellucida. These follicles are now designated as primary follicles. There is no evidence that gonadotrophic hormones are required to sustain follicles with less than four layers of granulosa cells since the initial phases of the growth process continue after removal of the pituitary gland in mice (Jones and Krohn, 1961). Beyond the four-layer stage, however, follicular growth is hormone dependent and involves further multiplication of granulosa cells to form the secondary follicle (Richards, 1980). The tertiary follicle or antral follicle develops with passage of fluid into the intracellular space (Baker, 1982). The mature preovulatory follicle or Graafian follicle is generally 10 mm or more in diameter and originates from a tertiary follicle.

In the cow, antrum formation starts in follicles ranging from 0.12 to 0.16 mm (Monniaux et al., 1984; Maurasse et al., 1985; Mariana and Machado, 1976; Dufour and Roy, 1985). The growth of antral follicles was divided into two phases by Lussier et al. (1987). The first phase involved early follicular growth due to an increase in the number of granulosa cells. The second phase occurred for follicles larger than 2.5 mm and was due primarily to increased antrum development rather than an increase in the number of granulosa cells. Lussier et al. (1987), by using the granulosa cell mitotic index on Day 14 of the cycle, estimated that a follicle requires 42 days of growth through the antral phase (0.13 mm to preovulatory size). Growth rate of antral follicles, however, varies with the size of the follicle as well as stage of follicular wave growth and the presence of a dominant follicle.

Patterns of follicular growth during the estrous cycle. During the bovine estrous cycle, several follicular waves can occur and each wave can be divided into three phases: selection, dominance and ovulation or atresia of

the dominant follicle (Ireland and Roche, 1987). The selection phase refers to recruitment of a single antral follicle from a population of many antral follicles for further growth. This dominant follicle is larger than any of the other follicles and is responsible for most of the estradiol secretion. In the final phase of dominant follicle development, the follicle will either ovulate (if luteolysis occurs) or will go through atresia (if the CL is maintained). Cows generally ovulate a single oocyte with double ovulations occurring with a frequency of 0.5 to 2.0% (Robinson, 1977).

Recent studies using real-time ultrasonography have shown that 70-80% of bovine estrous cycles are characterized by three waves of antral follicular growth (Fortune et al., 1988; Savio et al., 1988). Three waves of follicular growth have also been described by Ireland and Roche (1987) in which concentrations of estradiol in the utero-ovarian veins were monitored. This implies that about every seven days a large dominant follicle emerges from a pool of smaller follicles. The first dominant follicle is detected on Day 4-6 and correlates with the estradiol rise occurring during that time (Glencross et al., 1973). A second dominant follicle arises around Day 12-16 and plays an important role in initiating luteolysis. Finally, the third dominant follicle or preovulatory follicle is identified by Day 16 and reaches its maximum size on Day 21, just before ovulation (Pierson and Ginther, 1988; Savio et al., 1988). These latest findings are also in agreement with results of earlier studies in which the largest and second largest follicles were marked with India ink (Dufour et al., 1972; Matton et al.,1981). These researchers found that the preovulatory follicle cannot be predicted prior to 4 days before ovulation.

In some cases, a three-wave pattern of follicular development is not seen. Savio et al. (1988) reported that 4% and 15% of estrous cycles had only one and two dominant follicles,respectively, whereas Fortune et al. (1988)

described 20% of estrous cycles with two and 10% with four follicular waves. The follicular wave theory is not a new concept, since in 1960, Rajakoski used histological analysis to describe two waves of follicular growth; one between Day 3 and 12 and the other between Day 12 and subsequent estrus. Pierson and Ginther (1984, 1986), Ginther et al. (1989) and Knopf et al. (1989) have also described a two-wave cycle using ultrasonography. Knopf et al. (1989) detected the two-wave cycle in 90% of the examined heifers.

Follicular receptors and the two-cell theory of estrogen secretion. The regulation of follicular growth is dependent upon a close interrelationship between the hypothalamo-pituitary axis and the ovaries. The hypothalamus releases a gonadotropin-releasing hormone (GnRH) which binds to pituitary receptors to induce the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) into the peripheral blood circulation. These gonadotropins (FSH and LH) then reach the ovaries and act on ovarian follicles and the CL.

Prior to describing the endocrine control of follicular growth, it is necessary to first characterize the receptor population on follicular tissues as well as their impact on steroidogenesis. Richards (1980), using the rat as a model, demonstrated that preantral granulosa cells possess FSH receptors and do not acquire LH receptors until the antral stage. Theca cells from antral follicles contain LH receptors and never gain FSH receptors. In the cow, theca cells seem to contain LH receptors at all follicular stages. Granulosa cells of non-atretic follicles have receptors for FSH whereas only granulosa cells of large (> 6 mm) non-atretic follicles contain LH receptors (Ireland and Roche, 1982a, 1983a, 1983b). Under basal secretion of gonadotropin, theca cells respond to LH by synthesizing androgens (Erickson et al., 1985). Androgen secretion increases with LH, but not FSH (Hansel and Fortune, 1978).

Granulosa cells, which produce a very small quantity of androgens, are dependent upon thecal secretion of androgen as the substrate in estradiol synthesis (Lacroix et al., 1974; Armstrong and Dorrington, 1977).

The close interaction between granulosa and theca cells for estradiol production is recognized as the two-cell theory. Falck (1959) was the first one to provide evidence of a close interplay between these two cells types. He demonstrated that auto-transplantation of both granulosa and theca cells into the anterior chamber of the eye induces estrogen secretion whereas pure cell transplants did not. The biochemical basis of this theory emerged in 1966 when it was discovered that human theca cells are important producers of C_{19} androgens while the granulosa cells are the primary site of aromatase activity (Ryan and Petro, 1966). Lacroix et al. (1974) supported these findings by doing in vitro cell culture on bovine follicular tissues. They found that theca cells possess the enzymatic system that transforms pregnenolone to androstenedione, mainly through the Δ^5-pathway. The capacity to transform androstenedione to estrogens was very small in the thecal tissue, whereas granulosa cells were very efficient in aromatizing androstenedione to estradiol. The two-cell theory for the biosynthesis of estrogens was further developed by Armstrong and Dorrington (1977) to a more complete model called the two-cell two-gonadotropin model. Cultured granulosa cells obtained from ovaries of immature intact or hypophysectomized rats, when incubated with testosterone and FSH, produced large amounts of estrogen, whereas LH stimulation had essentially no effect when provided alone, or in combination with testosterone or FSH. Conversely, cultured thecal tissue isolated from proestrous rat follicles when incubated with LH produced approximately the same amount of radiolabelled androgens as when incubated with LH and FSH. Thus, the proposed model was that LH acts

upon the theca cells at an early biosynthetic step to stimulate androgen production. Granulosa cells, under the influence of FSH, aromatize the thecal androgens to estrogens.

These findings have been utilized in the elaboration of a generalized theory on follicular selection using the rat as a model (Richards, 1980; Richards and Hedin, 1988). During the rat estrous cycle, the growth of preovulatory follicles requires increased estradiol synthesis by the granulosa cells of antral follicles. This event depends upon the increased production of thecal-derived androgen as well as the increased responsiveness of theca cells to LH receptor population. Higher circulating levels of estrogens then stimulate release of more pituitary LH and FSH. The synergy of estrogens with gonadotropins stimulates the growth of a preovulatory follicle. The preovulatory follicle acquires LH receptors on its granulosa cells. The increased FSH responsiveness on the granulosa cells may reflect an increased number of granulosa cells per follicle or perhaps an increased effect of intrafollicular estradiol on the adenylate cyclase system (Richards et al., 1979; Richards and Hedin, 1988).

Cyclic changes in gonadotropins. In the cyclic cow, LH fluctuates in a pulsatile manner with the pattern varying with period of the estrous cycle (Rahe et al., 1980; Walters et al., 1984; Walters and Schallenberger, 1984). In the early luteal phase (Day 3-4), LH pulses are characterized by a high frequency (16-30 pulses/day) and low amplitude (0.3-1.8 ng/ml). During the midluteal phase (Day 9-14), LH pulse frequency (6-8 pulses/day) decreases and pulse amplitude (0.6-7.0 ng/ml) increases (Rahe et al., 1980; Walters et al., 1984). On Day 18-19 of the cycle, secretion of LH undergoes a preovulatory surge characterized by a high LH pulse frequency causing a rapid increase in baseline concentrations of LH (Rahe et al., 1980). Walters and Schallenberger

(1984) induced luteolysis in a group of cows and demonstrated that the preovulatory surge of LH occurred 59 h after administration of $PGF_{2\alpha}$-analogue and lasted 6-10 h. In addition, the preovulatory LH surge was the result of a simultaneous increase in the pulse frequency (i.e., decreased pulse interval from 200 min for diestrus to 38-40 min for estrus) and amplitude (7-32 ng/ml).

The pulsatile secretory pattern of LH is dependent upon the concentrations of secreted steroids. Progesterone has a negative feedback effect on the hypothalamo-pituitary axis and decreases GnRH and LH release (Convey et al., 1977; Roche and Ireland, 1981). Ireland and Roche (1982b) found that progesterone inhibits LH pulse frequency, but does not in itself affect the pulse amplitude. Thus, declining levels of progesterone result in an increased frequency in LH pulses (Ireland and Roche, 1982b; Imakawa et al., 1986).

Conversely, estrogen has been shown to be positively correlated with LH pulses during both the luteal and preovulatory phase of the bovine estrous cycle (Walters and Schallenberger, 1984; Walters et al., 1984). This is not surprising since estradiol secretion by follicles is directly related to LH concentrations as explained previously. Estradiol does not exert a positive feedback effect on the hypothalamo-pituitary axis in cows bearing a functional CL (Short et al., 1973). During the preovulatory period, however, the increasing secretion of estradiol, in association with low progesterone concentrations, is the stimulus that triggers the LH surge. Immunization against estradiol prevents the LH surge in cattle (Martin et al., 1978). In addition, injection of estradiol to ovariectomized heifers caused an increase in LH secretion without stimulating FSH secretion (Hansen et al., 1982).

Prior to the preovulatory LH surge, the increasing endogenous secretion of estradiol enhances the capacity of the pituitary gland to release LH in response to exogenous GnRH (Kaltenbach et al., 1974; Kesner et al., 1981). Estradiol also increases the ability of GnRH to release LH by bovine pituitary cell cultures, whereas progesterone inhibits this effect (Padmanabhan et al., 1982). Increasing dosages of estradiol administered to ovariectomized cows, caused an increase in the amplitude of LH pulses compared to those cows receiving low concentrations of estradiol, with no effect on LH pulse frequency (Kinder et al., 1983; Stumpf et al., 1989). The inability to detect an effect on LH pulse frequency may be due to the 10 min sampling interval. Walters and Schallenberger (1984) were unable to detect LH pulses in blood samples collected every 20 min; in contrast distinct pulses were observed when sampling was performed every 5 min. In intact cows during the preovulatory phase of an induced cycle, LH pulse frequency increases with no amplitude modification but LH pulse frequency and amplitude increase during the LH surge (Walters and Schallenberger, 1984).

Secretion of FSH is not well understood and seems to be regulated by a follicular peptide known as inhibin. Secretion of FSH is released in a pulsatile manner in the cow (Ireland and Roche, 1982b; Schallenberger and Peterson, 1982). Pulses of FSH could be secreted separately or in parallel with LH pulses. In the early luteal phase, the frequency of pulses is similar between FSH and LH, but in midcycle, the FSH pulse frequency is greater than that of LH (Walters et al., 1984). With the onset of luteolysis, estradiol increases within 4 to 6 h (Schallenberger et al., 1984; Walters et al., 1984) and LH within 1 to 3 h (Schallenberger et al., 1984) but no changes in FSH are observed before 12 h (Ireland et Roche, 1982a; Schallenberger et al., 1984). Following the ovulatory surge FSH increases on Day 4 of the cycle. This peak

lasts for 2 to 3 days (Ireland and Roche, 1983a; Schams et al., 1977). This second rise in FSH, which occurs without a concomitant peak in LH, may be due to removal of the negative effect of inhibin on the pituitary following ovulation (Hansel and Convey, 1983).

Bovine follicular fluid (bFF) contains inhibin-like activity (de Jong and Sharpe, 1976) that suppresses FSH secretion without affecting LH secretion in a bioassay using pituitary cells in culture (Padmanabhan et al., 1984). Ireland et al. (1983) demonstrated that the inhibin activity cannot be removed from charcoal-treated bFF from follicles larger than 10 mm, thus suggesting its proteinaceous nature. Infusion of bFF to ovariectomized heifers decreased FSH secretion without altering the LH secretion (Ireland et al., 1983; Beard et al., 1989) by directly inhibiting pituitary expression of the gene encoding for FSH-β subunit (Beard et al., 1989). From 0-36 h after $PGF_{2\alpha}$-induced luteolysis, inhibin activity increases in the dominant follicle. In the utero-ovarian vein, inhibin activity decreases until the preovulatory LH surge when it increases again until ovulation (Padmanabhan et al., 1984). Inhibin-like activity in atretic follicles is constant during the estrous cycle but is lower than in dominant follicles (Padmanabhan et al., 1984). Either inhibin-like activity (Ireland and Roche, 1987) or the effects of estradiol on FSH secretion (Hansen et al., 1982) may explain why there is no increase in FSH in the peripheral circulation before 12 h after luteolysis. Ireland and Roche (1987, p 10) hypothesized that "the absence of a transient rise in FSH in blood after luteolysis may also explain why only a single follicle ovulates after estrus in a cow".

Bovine inhibin (56-58 kDa molecule) is a heterodimer composed of an α subunit (43-44 kDa) and a β_A subunit (14-15 kDa) bound together by disulfide bonds (Robertson et al., 1985; Forage et al., 1986). A 31 kDa form of

bovine inhibin has been described by Forage et al. (1986) as being derived from proteolytic processing of the α (20 kDa) and β_A (15 kDa) subunits. Gene cloning of inhibin indicated that both subunits are products of different genes (Forage et al., 1986) and that the β subunit is a member of the transforming growth factor-β (TGF-β) family (Mason et al., 1985). Inhibin is secreted principally by granulosa cells in cows (Henderson and Franchimont, 1983), humans and pigs (diZerega et al., 1983a; Erickson and Hseuh, 1978). In situ hybridization, however, detected messenger ribonucleic acid (mRNA) for the α subunit in granulosa cells of antral follicles > 0.36 mm while the β_A subunit mRNA was present only in granulosa cells of follicles > 0.80 mm (Torney et al., 1989). In addition, the α subunit mRNA hybridized with the thecal layer of follicles > 0.80 mm. In pigs, the two isolated inhibins have identical α subunits and distinct β subunits (subunit β_A and β_B) (Ling et al., 1985; Mayo et al., 1986). When one β_A and one β_B subunit (Ling et al., 1986) or two β_A subunits (Vale et al., 1986) are paired together, the resulting molecule stimulates FSH release without affecting LH release. These two compounds, $\beta_A\beta_B$ and $\beta_A\beta_A$, have been named activin and activin-A (Ying, 1988). An activin binding protein has been identified in rat granulosa cells (Sugino et al., 1988; Nakamura et al., 1990) and bovine pituitary cells (Nakamura et al., 1990). In addition, activin binding protein seems to be identical to follistatin (Nakamura et al., 1990), another inhibitor of FSH release. Follistatin, a distinct peptide from inhibin and activin, has been isolated from porcine follicular fluid as a single peptide chain (Ueno et al., 1987). Like inhibin, follistatin suppresses release of FSH, but not LH release, from cultures of pituitary cells (Ueno et al., 1987). However, the significance of activin and follistatin on FSH regulation and ovarian functions remains to be determined.

Selection of the dominant follicle. Matton et al. (1981) presented evidence that in the cow the dominant follicle inhibits growth of smaller follicles. Cauterization of all follicles larger than 5 mm at various stages of the cycle stimulated a rapid development of medium-sized follicles into large-sized follicles. Fluctuations in gonadotropin secretion and presence of intraovarian factors could explain the ability of a dominant follicle to survive in an environment where growth of other follicles is suppressed. Hansel and Convey (1983, p 406) wrote that "changes in the pattern of episodic fluctuations in gonadotropin secretion may be related to follicular dynamics but . . . possibly, intraovarian factors play a role in development and turnover of follicles".

Dominant follicles secrete inhibin and follicular regulatory protein (FRP) into follicular fluid (Ireland and Roche, 1987). Ireland and Roche (1987) theorized that inhibin blocks development of other follicles by suppressing FSH secretion while FRP blocks, in a paracrine fashion, estradiol synthesis in non-dominant follicles resulting in their atresia. The FRP molecule is recognized as suppressing aromatase activity in porcine granulosa cells in vitro (diZerega et al., 1983b) and decreasing serum concentrations of estradiol in the primate (diZerega and Wilks, 1984). Bovine atretic follicles cannot secrete significant amounts of estradiol (Ireland and Roche, 1982a; Bellin and Ax, 1984), have two times FRP activity in follicular fluid (Ireland and Roche, 1987) and have higher levels of chondroitin sulfate-B in follicular fluid (Bellin and Ax, 1984) than estrogen-secreting follicles. Thus, the continuous secretion of estradiol by the dominant follicle enhances its sensitivity to gonadotropins and ensures its survival. Other intraovarian peptides known as GnRH-like peptides may also act in a paracrine manner to affect the development of ovarian follicles. The GnRH molecule exhibits

antigonadotropic actions in rat and porcine granulosa cells by suppressing FSH and LH stimulation of progesterone synthesis through inhibition of cAMP accumulation (Massicotte et al., 1980; Hsueh and Jones, 1981). Ovaries from cows, sheep and rats do not contain GnRH but contain GnRH-like proteins that compete with GnRH for binding to rat membrane receptors (Aten et al., 1986, 1987). Bovine GnRH-like proteins also suppress FSH stimulation of rat granulosa cells by inhibiting cAMP accumulation and progesterone synthesis (Aten and Behrman, 1988). These proteins are distinct from GnRH as they do not cross-react with specific antibodies to GnRH and elute differently on reverse phase high pressure liquid chromatography (HPLC) (Aten et al., 1986). The GnRH-like proteins are highly concentrated in bovine granulosa cells but significant amounts are also present in bovine luteal cells (Ireland et al., 1988). Nonetheless, the exact role of GnRH-like proteins in ovarian function remains to be determined since bovine and ovine ovaries do not contain GnRH receptors (Brown and Reeves, 1983).

The mechanism involved in atresia of a dominant follicle is still unknown. When a dominant follicle undergoes atresia, there is a decrease in follicular fluid estradiol and an increase in progesterone and androgens (Ireland and Roche, 1982a, 1983a, 1983b; Bellin and Ax, 1984). It was then speculated that if ovulation does not occur, the dominant follicle continues to secrete FRP which accumulates in the follicular fluid, thus decreasing its own estrogen production and causing atresia (Ireland and Roche, 1987).

Development of the Corpus Luteum (CL)

In 1573, Coiter, a student of Fallopius, first described the corpus luteum (see Asdell, 1977). Later, in 1672, de Graaf characterized the ovarian "globular bodies" as structures developing in place of the "ova" after ovulation. However, the term corpus luteum (CL), so named because of its yellow appearance in the cow, was introduced by Malpighi in 1697 (see Short, 1977). The CL is a transient endocrine gland that develops rapidly from the ovulated follicle. If fertilization does not occur, the CL regresses to allow growth of a new preovulatory follicle. The demise of the CL will be described in more details under "Luteolysis of the Cyclic CL".

Donaldson and Hansel (1965a), used histological examination to determine that most follicles begin early luteinization of the theca interna and granulosa layers 6 h after the onset of estrus in the cow. The preovulatory LH surge stimulates granulosa cells to differentiate into luteal cells that have the capacity to synthesis progesterone (Keyes and Wiltbank, 1988). In cows, luteinization of granulosa cells is associated with loss of androgen-aromatizing ability (Henderson and Moon, 1979). Theca interna cells also respond to plasma LH by multiplying and increasing in size to become large luteal cells (Donaldson and Hansel, 1965a). After ovulation, walls of the follicle collapse and 24-48 later, the basement membrane disappears and blood vessels from the theca interna invade the cavity of the ruptured follicle (Donaldson and Hansel, 1965a). Progesterone content and weight of the CL increase rapidly from Day 3 to 12 of the cycle and stay relatively constant until Day 16 (Erb et al., 1971). Studies (Priedkalns et al., 1968; Ursely and Leymarie, 1979; Koos and Hansel, 1981; Alila and Hansel, 1984; Chegini et al., 1984; Fields et al., 1985; Alila et al., 1988; O'Shea et al., 1989) have shown that the CL is composed of large luteal cells (> 25 μm in

diameter), small luteal cells (10-20 μm in diameter) and abundant smaller non-steroidogenic cells. The large luteal cells are characterized by having a round central nucleus with dispersed chromatin and small secretory granules. The secretory granules contain oxytocin (Wathes et al., 1983; Guldenaar et al., 1984; Fields et al., 1985; Rice and Thorburn, 1985; Sawyer et al., 1986; Theodosis et al., 1986), relaxin (Fields et al., 1985) and progesterone (Sawyer et al., 1979). These cells also contain numerous mitochondria and an abundance of smooth endoplasmic reticulum. The small luteal cells have acentric, cup-shaped nuclei with heterochromatin and contain large lipid droplets. A characteristic feature of the small luteal cells is the absence of secretory granules. In sheep, luteal tissues also consist of small and large cell types (O'Shea et al., 1980; Fitz et al., 1982; Rodgers and O'Shea, 1982).

In the morphological studies of Donaldson and Hansel (1965a), it was suggested that bovine granulosa cells differentiate into large luteal cells in the maturing CL. Based on the use of a monoclonal antibody to granulosa and theca cell surface antigens, Alila and Hansel (1984) provided evidence that small luteal cells of the early cyclic CL originate mostly from theca cells, while most of the large cells are derived from granulosa cells. In addition, as a consequence of exposure to LH, small cells may enlarge and replace granulosa-derived large cells in maturing CL. Alila and Hansel (1984) detected an increase in labeling of large luteal cells with a theca-specific monoclonal antibody, with approximately 10% of large luteal cells stained at Day 4-6 vs 46% at Day 10-12 of the cycle. Recently, morphometric analysis of the midcycle CL in the cow (O'Shea et al., 1989) estimated 103.3×10^6 small luteal cells and 13.6×10^6 large luteal cells per gram of luteal tissue at Day 12 of the cycle, with a ratio of small to large luteal cells of 7.6:1. In dispersed cell suspension of Day 10-12 cyclic CL (Hansel et al., 1987), cell number estimates

were 19.3 x 10^6 and 1.13 x 10^6 per CL for small and large luteal cells, respectively, with a small to large luteal cell ratio of 20:1-40:1. O'Shea et al. (1989) suggested that selective losses with a preferential loss of large luteal cells may occur during luteal cell dispersion.

Studies with hypophysectomized cows and ewes demonstrated clearly that the presence of pituitary tissue is necessary for the formation and maintenance of the CL (Kaltenbach et al., 1968; Denamur et al., 1973). The primary gonadotropin required for normal luteal development and function has been identified as LH in both species (Hansel et al., 1973; Niswender et al., 1986; Farin et al., 1987), though previous studies in the sheep caused controversy over whether prolactin is required in addition to LH (reviewed by Niswender et al., 1986). The number of bovine and ovine luteal receptors for LH increases as the estrous cycle progresses until the late luteal phase, when their number decline (Diekman et al., 1978; Garverick et al., 1985; Harrison et al., 1987). The number of unoccupied LH receptors is correlated with progesterone concentrations (Diekman et al., 1978; Garverick et al., 1985; Rao et al., 1979; Spicer et al., 1981). In cows, the concentration of unoccupied LH receptors increases more than four-fold from Day 4 to Day 16 and decreases thereafter (Garverick et al., 1985). Progesterone stimulates an increase in the number of LH receptors in bovine luteal cells from early but not midcycle (Jones et al., 1989).

Although large and small luteal cells produce progesterone in vitro, they have been shown to differ in their ability to secrete progesterone. In both cows and sheep, unstimulated large luteal cells secrete 6 to 10 times more progesterone than small luteal cells (Ursely and Leymarie, 1979; Koos and Hansel, 1981; Fitz et al., 1982; Rodgers and O'Shea, 1982; Harrison et al., 1987). In response to LH stimulation, however, small luteal cells secrete 6- to 20-fold

more progesterone than large luteal cells (Ursely and Leymarie, 1979; Koos and Hansel, 1981; Fitz et al., 1982; Harrison et al., 1987). The large cells have been shown to be unresponsive to LH, dibutyryl cAMP or other agents that stimulate cAMP while small luteal cells respond highly to LH in a cAMP-dependent manner (Fitz et al., 1982; Hoyer et al., 1984). Small luteal cells from superovulated ewes contain many LH receptors (~ 30 000) while large cells contain only a few receptors (~ 3 000; Fitz et al., 1982). In contrast, Harrison et al. (1987) found equal numbers of LH receptors on large and small ovine luteal cells on Days 10 and 15 of the estrous cycle of spontaneously ovulated ewes. The discrepancy between these two studies may depend on the source of luteal tissue (superovulated vs spontaneously ovulating ewes). The effect of equine chorionic gonadotropin on superovulated ewes has been reported to cause luteinization and loss of granulosa cells, leading to the development of a CL with hypertrophy of luteal-derived theca cells (Cran, 1983). Although the secretion of progesterone from large luteal cells is not directly regulated by LH, the number of large luteal cells may depend, at least in part, by differentiation of small to large luteal cells (Donaldson and Hansel, 1965a; Alila and Hansel, 1984).

The two types of steroidogenic luteal cells also differ markedly in other biochemical parameters. Receptors for estradiol are 5-fold more abundant in large ovine luteal cells (Glass et al., 1984). Fitz et al. (1982) detected approximately 32-fold and 12-fold more $PGF_{2\alpha}$ and PGE_2 receptors on large than on small ovine luteal cells. In addition, luteal cells from midluteal phase of superovulated ewes have high and low affinity binding receptors for $PGF_{2\alpha}$ (Balapure et al., 1989): small cells had low-affinity $PGF_{2\alpha}$ receptors whereas large luteal cells had both low and high-affinity sites. Prostaglandins $F_{2\alpha}$ and E_2 have been shown to stimulate progesterone secretion by bovine

luteal slices (Speroff and Ramwell, 1970). However, $PGF_{2\alpha}$ primarily enhances in-vitro secretion by progesterone of small bovine luteal cells (Hixon and Hansel, 1979; Benhaim et al., 1987; Alila et al., 1988), whereas PGE_2 primarily stimulates large luteal cells (Fitz et al., 1984a, 1984b; Silvia et al., 1984). Harrison et al. (1987) did not detect any effect of PGE_2 on stimulation of progesterone production by either large or small ovine luteal cells. The short exposure time of luteal cells to PGE_2 may explain these negative results. In cattle, $PGF_{2\alpha}$ binding to luteal membranes increased from Day 3 to Day 20 and decreased thereafter (Rao et al., 1979). During early stages of development, the CL contains predominantly small luteal cells and as the cycle progresses the number of large luteal cells increases to a maximum on Day 12 of the cycle (Niswender et al., 1985). These data suggest that during the early luteal phase, the CL is less responsive to $PGF_{2\alpha}$ than later in the cycle. This critical concept plays an important role at the time of luteolysis.

Luteal Function

The vital function of the CL is the secretion of progesterone which regulates the reproductive system (see next section). The CL also secretes several other hormones.

Large luteal cells of cattle and sheep synthesize oxytocin (Wathes et al., 1983; Guldenaar et al., 1984; Rice and Thorburn, 1985; Sawyer et al., 1986; Theodosis et al., 1986), with a maximal secretion rate during the early phase of the cycle (Rodgers et al., 1983; Harrison et al., 1987; Grazul et al., 1989). In mid-cycle in cattle, oxytocin concentrations in the vena cava were released in a pulsatile pattern which paralleled progesterone secretion (Walters and Schallenberger, 1984; Walters et al., 1984) while oxytocin paralleled $PGF_{2\alpha}$ during luteolysis (Schallenberger et al., 1984; Schams et al., 1985a, 1985b).

Schams (1987) suggested that oxytocin may play an important role in the regulation of progesterone early in the cycle and may later be involved in the luteolytic mechanism of $PGF_{2\alpha}$.

Bovine luteal cells also secrete GnRH-like peptides (Ireland et al., 1988). As explained earlier, GnRH-like peptides are chemically distinct from GnRH but share a similar antigonadotropic action on rat luteal and granulosa cells (Aten et al., 1986, 1987; Aten and Berhman, 1988). These GnRH-like proteins compete with GnRH for binding to rat granulosa and luteal receptors (Aten et al., 1986, 1987). The concentrations of GnRH-like peptides decrease during development of the bovine CL, but the greatest concentration is found in granulosa cells compared to other ovarian and non-ovarian tissues (Ireland et al., 1988). Ireland et al. (1988) suggested that a reduction in levels of GnRH-like proteins during CL development may reflect an enhanced capacity of the CL to respond to LH, as these peptides inhibit LH effects on rat luteal cells (Aten et al., 1987). However, this hypothesis is questionable in cattle since there are no GnRH receptors on bovine luteal cells (Brown and Reeves, 1983) and GnRH does not have an antigonadotropic effect on bovine luteal tissue (Milvae et al., 1984).

Luteal cells have recently been shown to secrete an angiogenic factor which may be important in development and function of the CL. Samples from corpus hemorrhagicum (Days 1-4), mid-cycle CL (Days 5-17) and late-cycle CL (Days 18-20) stimulate neovascularization in a bioassay, with an increase in response with age of the CL (Redmer et al., 1988). Late-cycle CL exerted the greatest angiogenic effect.

Role of Progesterone in Reproduction

During the estrous cycle in cattle, progesterone is secreted by large and small luteal cells (as described previously). During pregnancy, the CL remains the main source of progesterone, but bovine adrenal glands also contribute (1-4 ng/ml), especially after Day 200 of gestation (Wendorf et al., 1983). Early bovine embryos (Shemesh et al., 1979) and the definitive placenta (Ainsworth and Ryan, 1967) also secrete progesterone but in very small amounts. Thus, the bovine CL is essential during most, if not the entire period of pregnancy in order to prevent abortion. Removal of the CL prior to Day 200 of gestation results in fetal abortion (Wendorf et al., 1983; McDonald et al., 1953; Estergreen et al., 1967) while removal of the CL after Day 200 does not result in loss of the fetus due to secretion of progesterone by the adrenals (Hoffmann et al., 1979; Chew et al., 1979; Wendorf et al., 1983). In the ewe, continued secretion of luteal progesterone is required to maintain pregnancy until Day 50, after which the placental secretion of progesterone is adequate for the maintenance of gestation (Linzell and Heap, 1968).

In cyclic cattle, peripheral concentrations of progesterone are low (less than 0.5 ng/ml) from approximately 2 days preceding to 3 days following ovulation. Progesterone concentrations rise from Day 4 to about Day 12, peaking (4-8 ng/ml) by Day 14-18 of the cycle (Donaldson et al., 1970; Henricks and Dickey, 1970; Lemon et al., 1975; Chenault et al., 1975; Schams et al., 1977). The literature is contradictory as to whether progesterone concentrations at Days 10-18 post estrus differ between pregnant and cyclic or non-pregnant cows. In some studies, an increase in progesterone was detected in pregnant cows when compared to cyclic cows (Henricks and Dickey, 1970; Ford et al., 1979a; Lukaszewska and Hansel, 1980; Hansel, 1981; Lamming et al., 1989;

Schallenberger et al., 1989) while other studies failed to detect an effect of pregnancy on progesterone concentrations during this time (Shemesh et al., 1968; Donaldson et al., 1970; Sreenan and Diskin, 1983). After Day 16-18, progesterone concentrations remain elevated in pregnant cows but decline in cyclic cows as luteolysis proceeds (Henricks and Dickey, 1970; Lukaszewska and Hansel, 1980; Hansel, 1981; Donaldson et al., 1970). Between Days 130-150 of gestation, progesterone declines to 3-4 ng/ml and then increases to a maximum (7-8 ng/ml) at about Day 240 of gestation (Donaldson et al., 1970). During the last 20 to 30 days of gestation, progesterone decreases gradually and then falls drastically 2 to 3 days prior to parturition (Donaldson et al., 1970; Thorburn et al., 1977).

Progesterone plays a major role in regulation of the estrous cycle, fertilization, preparation of the uterus to receive an embryo and maintenance of pregnancy (Gomes and Erb, 1965). The following is a brief description of some of the effects mediated by progesterone on specific target organs of the female reproductive system.

High circulating levels of progesterone have a negative feedback effect on the bovine hypothalamo-pituitary axis by suppressing GnRH and LH release (Convey et al., 1977; Roche and Ireland, 1981; Ireland and Roche, 1982b). Thus, elevated progesterone concentrations in cyclic and pregnant females prevent ovulation and suppress sexual receptivity (Stabenfeldt et al., 1980). This principal effect of progesterone on the hypothalamo-pituitary axis is widely used in managing cattle reproduction to control the time of ovulation by administration of exogenous progestagens (Hunter, 1980).

During the first 16 days of the estrous cycle and of pregnancy, progesterone prepares the uterus to support the development of an embryo regardless of the presence or absence of a viable conceptus (Betteridge et al.,

1980, 1984). After Day 16, interaction of the conceptus with the maternal system prevents luteal regression, thus maintaining high levels of circulating progesterone. Progesterone inhibits endometrial cell division, induces a marked coiling of the endometrial glands and stimulates endometrial cells to secrete molecules necessary for the establishment of an histotroph milieu (Porter and Finn, 1977; Bazer and Roberts., 1984). Such an environment plays a critical role in the development of the conceptus until placentome formation (around Day 30 of gestation) (Renfree, 1982). Progesterone also causes the relaxation of endometrial smooth muscle and reduces myometrial activity (Lye and Porter, 1978).

Luteolysis of the Cyclic CL

For over 65 years, the uterus has been recognized as playing a critical role in controlling the lifespan of the CL in many species. Loeb (1923) was the first to discover that hysterectomy in the guinea pig prevents regression of the CL and prolongs the length of the estrous cycle. In cattle, surgical removal of the uterus extends the CL lifespan (Wiltbank and Casida, 1956; Anderson et al., 1962, 1965; Malven and Hansel, 1964). Similar results have been reported in sheep (Wiltbank and Casida, 1956; Anderson et al., 1969). In cattle and sheep, the uterine-luteal relationship occurs through a local instead of a systemic route. In both species, the CL is maintained when hemi-hysterectomy was ipsilateral to the CL but the CL regressed when the contralateral horn was removed (Inskeep and Butcher, 1966; Moor and Rowson, 1966c; Bland, 1970; Ginther et al., 1967; McCracken and Caldwell, 1969; Ginther, 1974, 1981; Mapletoft and Ginther, 1975).

The Uterine Luteolysin, $PGF_{2\alpha}$

It is well established that uterine $PGF_{2\alpha}$ is the endogenous mediator of luteolysis in sheep and cows. This concept is supported by several lines of evidence. The expected time of luteolysis coincides closely with increased concentrations of $PGF_{2\alpha}$ in uterine fluids (Wilson et al., 1972; Shemesh and Hansel, 1975; Ellinwood et al., 1979a; Vernon et al., 1981; Findlay et al., 1983), uterine flushes (Harrison et al., 1972; Ellinwood et al., 1979a; Lamothe et al., 1977; Bartol et al., 1981a; Findlay et al., 1983) and uterine venous drainage (Nancarrow et al., 1973; Thorburn et al., 1973; Shemesh and Hansel, 1975; Ellinwood et al., 1979a; Wolfenson et al., 1985; Hooper et al., 1986a, 1986b). Exogenous administration of $PGF_{2\alpha}$ and $PGF_{2\alpha}$-analogues mimics the luteolytic activity of endogenous $PGF_{2\alpha}$ (Hansel et al., 1973; Lauderdale, 1974; Thatcher and Chenault, 1976; King et al., 1982). In addition, indomethacin and immunization against $PGF_{2\alpha}$ prevent luteal regression (Scaramuzzi and Baird, 1976; Fairclough et al., 1976, 1981; Lewis and Warren, 1977).

Prostaglandin Synthesis. Arachidonic acid (a 20-carbon fatty acid) acts as precursors in the synthesis of prostaglandins (Horton and Poyser, 1976). According to Ramwell et al. (1977), phospholipids from cellular membranes are the major source of arachidonic acid for in-vivo synthesis of prostaglandins. The release of arachidonic acid from phospholipids is a limiting step in prostaglandin synthesis and is regulated by phospholipase A_2 (Ramwell et al., 1977). Free arachidonic acid is metabolized by cyclooxygenase resulting in the formation of endoperoxide (PGH_2), which is then acted upon by an isomerase, a reductase and a thromboxane synthetase to yield PGE_2, PGD_2, $PGF_{2\alpha}$ and thromboxane A_2 (Ramwell et al., 1977). Wlodawer et al. (1976) have shown that bovine endometrium contains the enzymes necessary for $PGF_{2\alpha}$ synthesis.

Lung, spleen and kidneys are the main sources of enzymes that metabolize $PGF_{2\alpha}$ to its main plasma metabolite, 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM) (Kindahl, 1980; Kindahl et al., 1984). The half-life of plasma $PGF_{2\alpha}$ is only a few seconds, whereas PGFM has an half-life of 7-8 min (Kindahl et al., 1976). Peripheral concentrations of PGFM are a reliable measure of $PGF_{2\alpha}$ release from uterine tissues (Kindahl et al., 1976, 1981, 1984; Guilbault et al., 1984; Knickerbocker et al., 1986c).

Counter-Current Mechanism. It has been postulated that the luteolysin leaves the uterus through the main uterine vein and then passes, by a counter-current exchange, directly into the ovarian artery for transport to the adjacent ovary (Vollmerhaus, 1964; McCracken et al., 1972). Morphological studies of bovine and ovine utero-ovarian vasculature demonstrated that the ovarian artery follows a tortuous path in close apposition to the wall of the utero-ovarian vein (Vollmerhaus, 1964; Del Campo and Ginther, 1974; Ginther and Del Campo, 1974), with the thinnest walls for both vessels in that area (Vollmerhaus, 1964; Del Campo and Ginther, 1974). In addition, inert gas, steroids and radiolabelled $PGF_{2\alpha}$ transfer directly from the utero-ovarian vein into the ovarian artery, but this is not the case for larger molecules such as bovine or human serum albumin (McCracken et al., 1972, 1984a; Coudert et al., 1974a, 1974b; Einer-Jensen and McCracken, 1981). Inert gas crosses very readily from the utero-ovarian vein to the ovarian artery whereas the transfer of steroids and $PGF_{2\alpha}$ are delayed by about 15 min (McCracken et al., 1972, 1984a; Coudert et al., 1974b; Einer-Jensen and McCracken, 1981). It has been estimated that 0.2-2% of $PGF_{2\alpha}$ is exchanged from the utero-ovarian vein to the ovarian artery (McCracken et al., 1972; Coudert et al., 1974b; Land et al., 1976; Wolfenson et al., 1985). Experiments with surgical anastomoses of uterine veins or ovarian arteries in

hemi-hysterectomized cattle and sheep have confirmed the existence of the veno-arterial pathway (Ginther, 1974, 1981; Mapletoft and Ginther, 1975).

Even though the counter-current mechanism is well accepted, other alternative routes for local $PGF_{2\alpha}$ transfer may exist. In cattle, sectioning of the ovarian artery distal to the area of veno-arterial apposition was unable to extend CL lifespan, suggesting the development of a compensatory or alternate mechanism (Lamond and Drost, 1973). Abdel Rahim et al. (1984) reported that 7 of 8 cycling ewes had extended luteal function when all tissues except the utero-ovarian vessels involved in the counter-current mechanism were disconnected between the uterus and the ovaries. There was also frequent failure to prevent CL regression when the utero-ovarian vein was mechanically separated from the ovarian artery or when the main uterine vein was ligated and severed (Kiracofe et al., 1966; Dobrowolski and Hafez, 1970; Thorburn and Mattner, 1971; Baird and Land, 1973). One alternative route for $PGF_{2\alpha}$ transfer may be via the oviductal vein (Alwachi et al., 1981). In sheep, high concentrations of $PGF_{2\alpha}$ were detected in the oviductal vein at Day 12-16 of the cycle. It was suggested that $PGF_{2\alpha}$ may be transferred by a counter-current exchange between the venous drainage passing alongside the oviduct and the ovarian artery.

Strong evidence supports the contribution of the lymphatic system in the transport of uterine $PGF_{2\alpha}$ to the ovary. In sheep, lymphatic ducts draining the uterus and ovaries form a common utero-ovarian network along the vascular pedicle, with a closer association with the ovarian artery rather than the utero-ovarian vein (Staples et al., 1982; Staples and Whylie, 1984). Prostaglandin $F_{2\alpha}$ was present in utero-ovarian lymphatic vessels at the time of luteolysis (Staples and Whylie, 1984) and was transferred from the uterine lumen into uterine lymphatic vessels (Heap et al., 1985; Heap et al.,

1989). After infusion of radiolabelled $PGF_{2\alpha}$ into a uterine lymphatic duct, more radiolabelled compounds were recovered in both ovary and CL adjacent to the site of infusion than after infusion into the uterine vein (Heap et al., 1985). Infusion of $PGF_{2\alpha}$ into a major uterine lymphatic vessel at Day 7-8 of the cycle induced regression of the CL (Staples and Whylie, 1984). Furthermore, ligation of the utero-ovarian lymphatic ducts extended the estrous cycle beyond 20 days in 28% of the ewes (Staples and Whylie, 1984).

Regulation of Uterine $PGF_{2\alpha}$ Production.

Endocrine regulation of $PGF_{2\alpha}$ is a complex phenomenon and involves the interaction of several ovarian and uterine hormones. Since uterine regulation of $PGF_{2\alpha}$ is similar for cows and sheep, the following discussion will not always delineate between species. It should be remembered that luteolysis occurs around Day 15 in the ewe and Day 17 in cattle (see Inskeep and Murdoch, 1980).

McCracken et al. (1981, 1984b), using the ewe as model, proposed a model for luteolysis which still appears to be valid for both the sheep and the cow. At the end of the luteal phase (at least 10 days of elevated progesterone), follicular secretion of estrogen increases and estradiol interacts with its receptor, inducing production of endometrial receptors for oxytocin. Then oxytocin, possibly from both luteal and pituitary origins, binds to endometrial receptors and stimulates release of $PGF_{2\alpha}$ by increasing frequency and amplitude of $PGF_{2\alpha}$ pulses. The initial release of $PGF_{2\alpha}$ acts upon the CL to decrease luteal production of progesterone and to release more luteal oxytocin to reinforce endometrial production of $PGF_{2\alpha}$. This cycle continues until large luteal cells are depleted of oxytocin. There are many studies supporting this model.

Administration of estrogens in midluteal phase of the estrous cycle stimulates uterine release of $PGF_{2\alpha}$, increases peripheral concentrations of PGFM (Barcikowski et al., 1974; Bartol et al., 1981b; Thatcher et al., 1984b, 1986b; Lafrance and Goff, 1985; Hixon and Flint, 1987) and causes regression of the CL (Thatcher et al., 1986b; Wiltbank, 1966; Brunner et al., 1969; Eley et al., 1979). Loy et al. (1960) injected cyclic cattle with estradiol from Day 1 to Day 13 and did not detect any effect on luteal production of progesterone, suggesting that early administration of estradiol does not induce premature luteolysis. Similar effects have been reported with oxytocin (Lafrance and Goff, 1985; Aulettaa et al., 1972; Milvae and Hansel, 1980a). In cattle, the injection of oxytocin at Day 17-19 of the estrous cycle caused an increase in peripheral concentrations of PGFM, but not at Days 6 and 13 (Lafrance and Goff, 1985). The uterus seemed to require at least 8-10 days of exposure to normal luteal concentrations of progesterone in order to initiate luteolysis (Brunner et al., 1969; Loy et al., 1960; Woody et al., 1967; Louis et al., 1977; Ottobre et al., 1980; Lafrance and Goff, 1988; Homanics and Silvia, 1988). Thus, administration of progesterone from Days 1 to 6 of the estrous cycle resulted in a short estrous cycle, due probably to precocious induction of the luteolytic system (Woody et al., 1967; Ottobre et al., 1980; Ginther, 1968; Ginther, 1969; Lawson and Cahill, 1983).

During the late phase of the estrous cycle, peripheral concentrations of estradiol increased just prior to a reduction in progesterone (Barcikowski et al., 1974; Fogwell et al., 1985). Destruction of ovarian follicles during mid-cycle suppressed estradiol production and extended luteal lifespan (Ginther, 1970; Nancarrow et al., 1973; Barcikowski et al., 1974; Fogwell et al., 1985; Hughes et al., 1987).

There is increasing evidence that endogenous oxytocin also plays an important role in luteolysis. The best evidence is certainly the extension of the luteal lifespan following immunization against oxytocin (Sheldrick et al., 1980; Schams et al., 1983a). During the estrous cycle, peripheral concentrations of oxytocin reflected the ability of the luteal cells to produce progesterone, both hormones falling at luteal regression and rising with the formation of a new CL (Webb et al., 1981; Schams et al., 1982; Flint and Sheldrick, 1983; Walters and Schallenberger, 1984; Walters et al., 1984). At the time of luteolysis, frequent blood samplings of the jugular vein or the utero-ovarian vessels detected synchronous surges of oxytocin and oxytocin-neurophysin with $PGF_{2\alpha}$ or PGFM concentrations (Fairclough et al., 1980, 1983; Flint and Sheldrick, 1982; Hooper et al., 1986b). In addition, following $PGF_{2\alpha}$ administration, there was a rapid decrease in progesterone and a brief increase in ovarian release of oxytocin (Flint and Sheldrick, 1983). Flint and Sheldrick (1983) suggested that the uterine release of $PGF_{2\alpha}$ stimulated oxytocin release from the ovary, which in turn, stimulated the release of uterine $PGF_{2\alpha}$.

In the corpus luteum, large luteal cells were primarily responsible for the production of oxytocin (Rodgers et al., 1983; Guldenaar et al., 1984) while in the developing follicle, granulosa cells synthesized small amount of oxytocin (Kruip et al., 1985; Schams et al., 1987). In the cow, luteal transcription of oxytocin mRNA peaked at Days 3-6 while cellular oxytocin content peaked on Days 11-18 of the estrous cycle (Schams et al., 1987).

The concentration of endometrial receptors for progesterone, estradiol and oxytocin also change during the estrous cycle. During midcycle, progesterone suppressed synthesis of its own receptors (Zelinski et al., 1982) and the estrogen receptors (Clark et al., 1977; Koligian and Stormshak, 1977a,

1977b). Concentration of endometrial receptors for oxytocin increased during luteolysis and peaked at estrus (Roberts et al., 1975, 1976; McCracken et al., 1981, 1984b; Sheldrick and Flint, 1985). When progesterone concentrations started decreasing at the end of the cycle, increasing secretion of follicular estrogens were associated with the synthesis of estrogen (Clark et al., 1977; Koligian and Stormshak, 1977a, 1977b) and oxytocin receptors (Roberts et al., 1975, 1976; McCracken et al., 1981, 1984b; Sheldrick and Flint, 1985).

New insights have indicated that oxytocin increases endometrial production of $PGF_{2\alpha}$ by stimulating the phosphatidyl inositol turnover on the cell membrane (Hixon and Flint, 1987; Flint et al., 1986; Vallet and Bazer, 1989; Mirando et al., 1990). Oxytocin, by binding to its receptor, promotes hydrolysis of phosphoinositides, thus, releasing more diacylglycerol which may be metabolized to yield arachidonic acid. This mechanism increases the amount of arachidonic acid available for $PGF_{2\alpha}$ synthesis.

It is still unclear which event is triggering the luteolytic mechanism. Is the initial decline in circulating progesterone the cause or effect of luteolysis? There are three events that occur around luteolysis which could be responsible for the first decrease in progesterone (functional luteolysis of the CL) and may also play critical roles in structural luteolysis (degradation of the luteal tissue).

There is evidence that the release of few $PGF_{2\alpha}$ pulses precede a noticeable decline in progesterone (Thorburn et al., 1973; Hooper et al., 1986a; Zarco et al., 1988b). In sheep, Zarco et al. (1988b) detected 2.1 pulses of $PGF_{2\alpha}$ (estimated by measurement of PGFM and 11-ketotetranor-PGF metabolites) prior to an initial decline in peripheral concentrations of progesterone; 6.3 pulses were associated with functional luteolysis. An increase in $PGF_{2\alpha}$ would then inhibit progesterone production by the large luteal cells (Silvia et

al., 1984). Large ovine luteal cells contained most of the $PGF_{2\alpha}$ receptors (Fitz et al., 1982), which were constituted of low-affinity and high affinity binding sites (Balapure et al., 1989). On the other hand, small luteal cells contain low-affinity binding receptors for $PGF_{2\alpha}$ and should be less responsive to higher pulsative levels of $PGF_{2\alpha}$ (Fitz et al., 1982; Balapure et al., 1989) This concept is important since in culture $PGF_{2\alpha}$ suppressed the secretion of progesterone by large luteal cells but increased it when incubated with small luteal cells (Silvia et al., 1984). In addition, $PGF_{2\alpha}$ decreased the viability of large but not small luteal cells (Silvia et al., 1984).

Blood flow and blood pressure in the ovarian artery ipsilateral to the CL have been shown to parallel the fluctuations in progesterone concentrations; increasing during luteal development and decreasing during luteolysis (Hooper et al., 1986a; Niswender et al., 1976). Intrauterine injection of $PGF_{2\alpha}$ decreased blood flow in the ovarian artery on the side of the CL by 4 h followed by a decrease in peripheral concentrations of progesterone 2 h later (Niswender et al., 1976). Since approximately 95% of the blood flow is directed to the CL (Niswender et al., 1976; Ford et al., 1979b), the decrease in blood flow of the ovarian artery may contribute to the process of luteolysis by depriving the CL of essential nutrients. However, Einer-Jensen and McCracken (1981) did not detect a reduction in the capillary blood flow through the ovine CL after a direct injection of $PGF_{2\alpha}$ into the CL, but did detect a decline in progesterone concentrations 2 h post-injection. The decline in ovarian blood flow may not be the triggering event in luteolysis but could still be important in structural luteolysis.

There is increasing evidence that cells of the immune system may be involved in the process of luteolysis. In the cow, lymphocytes start migrating into the CL at Day 14 (Lobel and Levy, 1968). Bovine luteal cells also express

class I major histocompatibility antigens (MHC; cell-mediated cytotoxic reaction) and a limited amount of class II MHC (antigenic presentation) (Fairchild and Pate, 1989). In sheep, injection of $PGF_{2\alpha}$ at Day 10 of the cycle elicits luteal secretion of a chemoattractant for eosinophils which stimulates eosinophil migration into the CL (2 h post-injection) before a decrease in circulating concentrations of progesterone (3-4 h) and luteal weight (8 h) (Murdoch, 1987). It has been suggested that $PGF_{2\alpha}$ may act to uncover antigens on luteal cells and these antigens may then promote an autoimmune-like response (Murdoch, 1987). This process would result in the local accumulation of lymphocytes, eosinophils, macrophages and other immune cells. Eosinophils can lyse luteal cells by releasing cytotoxic secretory proteins (Gleich and Loegering, 1984) and macrophages can phagocytize or intact luteal cells (Paavola, 1979).

Establishment of Gestation

The Critical Period

High levels of progesterone are essential for the development of bovine and ovine embryos during at least the first one-third of pregnancy (Wendorf et al., 1983; McDonald et al., 1953; Estergreen et al., 1967; Linzell and Heap, 1968). In domestic ruminants, the early conceptus must signal its presence to the mother to prevent luteolysis. Three mechanisms have been elaborated to explain the possible effect of the conceptus on the CL. They are the antiluteolytic-anti-PGF, antiluteolytic-luteoprotective and luteotrophic mechanisms (Thatcher et al., 1986a). The antiluteolytic-anti-PGF mechanism refers to a decrease in the release of $PGF_{2\alpha}$ from the uterus at the expected time of luteolysis. The antiluteolytic-luteoprotective mechanism directly

protects the CL by decreasing the luteal response to $PGF_{2\alpha}$. The luteotrophic mechanism refers to an amplified secretion of progesterone by luteal tissue.

In cattle, pregnancy can be established after the synchronous transfer of conceptus up to 16 days after estrus but not afterwards (Betteridge et al., 1980, 1984). Removal of Day 17-19 bovine conceptuses, but not Day 13-15 conceptuses, and intrauterine infusion of Day 16-18 conceptus homogenates extend luteal lifespan and the length of the estrous cycle by 4-7 days (Northey and French, 1980; Humblot and Dalla Porta, 1984; Albihn, 1988). In sheep, similar results have been observed. Conceptus removal after Day 12 extends the interestrous interval by 6-7 days (24.5 days) as compared to embryos removed by Day 12 of pregnancy (18.0 days) (Moor and Rowson, 1966a). Moor and Rowson (1966b) reported that 65% of ewes receiving an embryo on Day 12 of the cycle became pregnant whereas only 12% of embryos transferred on Day 13-14 of the cycle were successful in establishing pregnancy. Intrauterine infusion of Day 14-16 ovine conceptus homogenates or extracts to cyclic ewes extended luteal lifespan for more than 6 days (Ellinwood et al., 1979b; Rowson and Moor, 1967; Martal et al., 1979). However, intramuscular administration of homogenates from Day 14-15 conceptuses or intrauterine infusion of Day 25 conceptus homogenates did not alter the cycle length (Rowson and Moor, 1967).

The process by which the maternal physiology changes in response to the presence of the conceptus has been termed "maternal recognition of pregnancy" (Short, 1969). One of the most striking and most study aspects of this process has been the extension of the luteal lifespan. Thus, the critical period in establishment of pregnancy appears to be between 15-17 days after estrus in the cow and 12-13 days after estrus in the ewe. Nonetheless, the conceptus may alter the maternal physiology at an earlier stage, but the lack of

reliable biological markers (as compared to CL lifespan) make this determination more difficult.

Unilateral Relationship between the Gravid Horn and the CL

Experimental evidence suggests that bovine and ovine conceptuses prevent regression of the CL through a local pathway (Mapletoft et al., 1975, 1976; Del Campo et al., 1977, 1980). In ewes, experiments using cross-vascular anastomosis showed that the isolated gravid horn exerts a local effect on the CL by sending a message, from either the conceptus or the endometrium, into the main uterine vein to the ovarian artery (Mapletoft et al., 1975, 1976). In cows, transfer of an embryo into an isolated horn ipsilateral to the CL extends lifespan of the CL until at least 24 days whereas transfer of embryos into the contralateral horn results in luteolysis (Del Campo et al., 1977). Moreover, anastomosis between the uterine vein ipsilateral to the gravid horn and the opposite uterine vein, ipsilateral to the CL and surgically isolated from the rest of the uterus, results in maintenance of the CL until at least 24 days (Del Campo et al., 1980).

It is possible that alternative pathways exist between the gravid horn and the CL. At least in the pig, there is preliminary evidence that the lymphatic system may play a role in early recognition of pregnancy (Magness and Ford, 1982).

Maternal Changes during Early Pregnancy

Fluctuations of circulating hormones. According to Schallenberger et al. (1989), pulse frequency of progesterone and gonadotropins are similar in early pregnant and cyclic cows whereas the amplitude of LH and progesterone increase between 2 and 4 weeks of gestation. Previous studies have also

detected an increase in progesterone concentrations in early pregnancy (Henricks and Dickey, 1970; Ford et al., 1979a; Lukaszewska and Hansel, 1980; Hansel, 1981; Lamming et al., 1989). Detection of higher levels of progesterone in pregnant animals is in support of a luteotrophic action of the embryo on the CL.

Between Days 6 and 18, jugular estradiol concentrations are higher in pregnant as compared to cyclic cows (Lukaszewska and Hansel, 1980; Hansel, 1981). Recently, Schallenberger et al. (1989) reported lower estradiol concentrations in the aorta of pregnant cows when compared to cyclic cows, from Days 4-7 to at least Day 35. The same authors, however, detected an increase in estradiol concentrations in the posterior vena cava after Day 14 of gestation and ascribed this to a uterine source rather than an ovarian one by positioning the catheter in various location within the vena cava. Nonetheless, even small changes in estradiol production may play a role in the inhibition of luteolysis by altering the uterine blood flow (Ford et al., 1979b; Schallenberger et al., 1989; Thatcher et al., 1986b; Reynolds, 1986).

In pregnant cows, circulating concentrations of oxytocin are lower at Days 4-7 compared to cyclic animals (Schallenberger et al., 1989), possibly due to a decrease in the oxytocin content of the pregnant CL (Schams et al., 1983b; Wathes et al., 1983). Using hourly bleeding in ewes, a decrease in the pulsatile release of oxytocin and neurophysin-oxytocin at Day 13-16 of pregnancy was detected as compared to cyclic animals (Webb et al., 1981; Moore and Watkins, 1982). Sheldrick and Flint (1983) also reported a decrease in peripheral concentrations of oxytocin at Days 10-15 of gestation and a decline in luteal concentrations of oxytocin. In another study, pulse frequency did not differ at Days 13-16 between pregnant and cyclic ewes but the peak height and amplitude was smaller in pregnant animals (Hooper et al., 1986b).

Frequency and amplitude of PGFM pulses in the peripheral circulation (Kindahl et al., 1976; Thatcher et al., 1984b; Betteridge et al., 1984; Basu and Kindahl, 1987a) as well as amplitude of PGF spikes in the ovarian artery ipsilateral to the gravid horn and CL (Wolfenson et al., 1985) are reduced in pregnant cows at Day 17-20 post estrus. However, the basal concentration of peripheral PGFM has been reported to be similar (Lafrance and Goff, 1985; Rico et al., 1981; Putney et al., 1989b) or to be higher in pregnant rather than cyclic cows (Basu and Kindahl, 1987a; Williams et al., 1983). At Day 13-16 post estrus, pregnant sheep have reduced frequency and amplitude pulses of PGFM in the peripheral circulation (Webb et al., 1981; Peterson et al., 1976; Nett et al., 1976; Lacroix and Kann, 1986; Zarco et al., 1988a) and PGF in utero-ovarian vessels (Thorburn et al., 1973; Hooper et al., 1986a, 1986b). Basal concentration of PGFM, however, is higher in pregnant ewes at Day 13-17 after estrus (Zarco et al., 1988a; Fincher et al., 1986).

Modification of the utero-ovarian blood flow. In cattle, between Days 14 and 18, blood flow in the uterine artery ipsilateral to the gravid horn increases two to three fold while blood flow to the non-gravid horn remained constant (Ford et al., 1979a). In another study performed on a single pregnant cow, there was a three-fold increase in blood flow in the ovarian artery ipsilateral to the gravid horn (Ford and Chenault, 1981). These results suggest that the conceptus, by altering the blood flow to the gravid horn, may increase the perfusion rate of the ovary, thus helping luteal cells to secrete more progesterone and/or enhancing the delivery of a luteoprotective or luteotrophic factor to the CL (Thatcher et al., 1986a).

Follicular development. As determined by ultrasonography, the dominant follicle in pregnant heifers begin to regress around Day 17 whereas it is maintained or increased in diameter in cyclic heifers (Pierson and

Ginther, 1987; Ginther et al., 1989). The growth of follicles larger than 7 mm decreases in the ovary bearing the CL, especially after Day 21-22 of gestation (Pierson and Ginther, 1986). Histological examination of the ovary bearing the CL at Day 17 after estrus also detected an effect of the conceptus on follicular development (Guilbault et al., 1986). Follicular turnover was more rapid for follicles classified from 0.16-0.28 mm to 0.68-3.67 mm on CL-bearing ovaries in pregnant as compared to cyclic cows, although further growth of follicles > 3.67 mm was limited due to atresia. Thus, the conceptus may alter follicular growth and reduce secretion of luteolytic levels of estradiol (Thatcher et al., 1989b).

Regulation of $PGF_{2\alpha}$ secretion. Endometrial tissues from pregnant cows at Day 17 post estrus synthesize less $PGF_{2\alpha}$ in vitro than those of cyclic cows as tested with either an explant culture system (Thatcher et al., 1984a) or a perifusion system (Gross et al., 1988c). In addition, perfused endometrium of pregnant cows does not respond to an oxytocin challenge, contrary to tissue from cyclic cows. The reduced response during pregnancy could be due to reduced oxytocin receptors, though endometrial concentrations of oxytocin receptors have not been reported in cattle. Data accumulated in sheep indicate that the number of receptors are lower during pregnancy (McCracken et al., 1984b; Flint et al., 1986). These results are in agreement with in-vivo studies. Pulsatile release of $PGF_{2\alpha}$ in the utero-ovarian vasculature and PGFM in the peripheral circulation are decreased in pregnant cows around the time of luteolysis (Kindahl et al., 1976; Wolfenson et al., 1985; Thatcher et al., 1984b; Betteridge et al., 1984; Basu and Kindahl, 1987a). The stimulation of PGFM caused by oxytocin (Lafrance and Goff, 1985) or estradiol administration (Thatcher et al., 1984b; Rico et al., 1981) is suppressed in pregnant cows as compared to cyclic cows.

The reduction in synthesis of PGF around Day 17 may result from the induction of an intracellular inhibitor of PGF synthesis (endometrial prostaglandin synthesis inhibitor = EPSI) (Basu and Kindahl, 1987b; Gross et al., 1988b). The inhibitor seems to be proteinaceous with 2 distinct Mr forms (25-35 kDa and 70-75 kDa) (Gross et al., 1988b). The EPSI, furthermore, inhibits production of PGF and PGE in a cell-free prostaglandin synthesizing system presumably suppressing cyclooxygenase activity (Gross et al., 1988b). Moreover, the uptake of $PGF_{2\alpha}$ by ovarian veins and ovarian arteries of pregnant cows is suppressed at Day 17 post estrus, which may help to reduce the amount of $PGF_{2\alpha}$ carried to the ovary bearing the CL (Thatcher et al., 1984b).

Endometrium from Day 13-16 pregnant sheep contains fewer oxytocin receptors compared to cyclic ewes (McCracken et al., 1984b; Flint et al., 1986). Frequency and/or amplitude of oxytocin and neurophysin-oxytocin pulses is also suppressed (Webb et al., 1981; Hooper et al., 1986b; Moore and Watkins, 1982; Sheldrick and Flint, 1983). These results may explain the inability of a pregnant ewe to secrete luteolytic levels of $PGF_{2\alpha}$. This is supported by the fact that an injection of oxytocin (Fairclough et al., 1984) or estradiol (Lacroix and Kann, 1986; Fincher et al., 1986; Kittok and Britt, 1977) at Day 14-19 of gestation fails to induce PGFM pulses and luteolysis as occurring in cyclic ewes. Contrary to resutls with cows, experiments with cultured endometrium from pregnant ewes indicate that PGF secretion is similar (Lewis et al., 1978; Lacroix and Kann, 1982) or higher than during the cycle (Ellinwood et al., 1979a; Findlay et al., 1981; Nett and Niswender, 1981). Endometrium from pregnant ewes contains more PGF than that from cyclic ewes (Ellinwood et al., 1979a; Wilson et al., 1972; Lewis et al., 1977; Findlay et al., 1983) and there were higher concentrations of PGF in uterine fluids

(Findlay et al., 1983). Lacroix and Kann (1983), using a perifusion device, demonstrated that secretion of PGF is higher at Day 14 and 17 of gestation than during the cycle, with a greater secretion of PGF toward the luminal than myometrial side. Similar results have been reported by Vallet et al. (1989) in which endometrial tissues from Day 14 pregnant and cyclic ewes were perifused.

In pregnant cows and ewes, there is a decrease in the pulsatile release of $PGF_{2\alpha}$ at the expected time of luteolysis, supporting the antiluteolytic-anti-PGF mechanism. This decrease in pulse frequency is not accompanied by a reduction in the basal secretion rate of $PGF_{2\alpha}$. Results from in vitro studies differ between these two species. In vitro culture of endometrial tissues from pregnant cows seems to simulate the in vivo situation related to pulsatile release of $PGF_{2\alpha}$, whereas data derived from the in vitro culture of endometrial tissues from pregnant sheep demonstrated either no decrease or an increase in PGF and may reflect baseline concentrations only.

Secretion of a Proteinaceous Signal by the Conceptus

As mentioned previously, intrauterine infusion of extracts and/or homogenates of conceptuses extends the luteal lifespan in cattle (Northey and French, 1980; Humblot and Dalla Porta, 1984; Albihn, 1988) and sheep (Ellinwood et al., 1979b; Rowson and Moor, 1967; Martal et al., 1979). The active component in ovine conceptus homogenates has been reported to be heat-labile, protease-sensitive, absent beyond Day 21 of gestation (Rowson and Moor, 1967; Martal et al., 1979) and to contain neither LH/hCG-like nor prolactin-like activity (Ellinwood et al., 1979b).

The active component, furthermore, was shown to be secreted by trophoblastic tissues (trophectoderm and endoderm) rather than the embryo

proper (Heyman et al., 1984; Plante et al., 1984). Trophoblastic vesicles from Day 14 bovine conceptuses or from Day 11-13 ovine conceptuses, when transfered to the specific species, extend the cycle for 8 of 12 recipient cows and 7 of 12 recipient ewes (Heyman et al., 1984). Reciprocal interspecies transfer of bovine and ovine trophoblastic vesicles into uteri of cyclic recipients also modified the CL lifespan, indicating that these two species secrete a similar signal for maternal recognition of pregnancy (Martal et al., 1984).

Intrauterine administration of proteins obtained from dialysed culture medium conditioned by bovine or ovine conceptuses extended cycle length in the specific species (Godkin et al., 1984b; Knickerbocker et al., 1986a). The total array of secreted proteins by Day 16-18 bovine or Day 15-16 ovine conceptuses is very often referred to as bovine and ovine conceptus secretory proteins (bCSP and oCSP).

Infusion of bCSP into the uterine lumen of cyclic cows, from Day 15.5 to 21 of the estrous cycle, extends the interestrous interval (33 days) and lifespan of the CL (30 days) (Knickerbocker et al., 1986a). Cows infused in utero with bCSP from Day 15 to 18 had lower release of PGFM in response to luteolytic dose of estradiol as compared to cows receiving dialysed serum protein (Knickerbocker et al., 1986b). Prostaglandin F secretion from endometrial explants collected at Day 17 of the estrous cycle was inhibited by bCSP and this decrease was accompanied by the induction of EPSI (Gross et al., 1988a). In addition, bCSP did not alter PGE_2 secretion but induced the secretion of several 13-14 kDa proteins (Gross et al., 1988a).

Intrauterine infusion of oCSP to cyclic ewes, from Days 12-18 post-estrus, prolongs the luteal lifespan for more than 23 days (Godkin et al., 1984b). Daily infusion of oCSP on Days 12-14 inhibits PGFM production

following an estradiol (Day 14) and an oxytocin challenge (Day 15) (Fincher et al., 1986).

These results suggest that bovine and ovine conceptuses secrete a proteinaceous molecule of trophectoderm-endoderm origin with antiluteolytic-anti-PGF activity.

Bovine trophoblast protein-1. Bartol et al. (1985a) characterized the two-dimensional electrophoretic pattern of nondialysable radiolabelled molecules in conditioned medium from culture of conceptuses from Day 16, 19, 22, 24 and 29, and chorion from Day 69. On Days 16 through 24, conceptuses secreted an array of prominent low-molecular weight acidic polypeptides (22-26 kDa) migrating in an isoelectric range of 6.5 to 5.6. Later, this complex of proteins was shown to cross-react immunologically with an antibody raised against ovine trophoblast protein-1 (oTP-1; a purified protein from oCSP that will be described in the following section) and was then called bovine trophoblast protein-1 (bTP-1) (Helmer et al., 1987). In situ hybridization using oTP-1 cDNA probes detects bTP-1 mRNA in trophectoderm of bovine conceptuses as early as Day 12 (Farin et al., 1989) although bTP-1 is secreted in larger quantities by conceptuses between Days 17-22 of gestation (Bartol et al., 1985a; Godkin et al., 1988c). During this period of substantial bTP-1 production, total production increases with elongation of the conceptus (Geisert et al., 1988a). The bTP-1 complex continues to be secreted in smaller amounts by the chorioallantois until at least Day 38 of gestation (Bartol et al., 1985a; Godkin et al., 1988a, 1988c). The bTP-1 complex can also be identified in uterine fluids from Day 15-25 pregnant cows (Kazemi et al., 1988). Immunocytochemical studies on Day 20 bovine conceptus have localized bTP-1 in the cytoplasm of both mono- and binuclear cells of the trophectoderm (Lifsey et al., 1989).

Biochemically, the bTP-1 complex consists of at least 7-9 isomers falling into three molecular weight classes (20 kDa, 22 kDa and 24 kDa) (Bartol et al., 1985a; Helmer et al., 1987, 1989b; Anthony et al., 1988; Geisert et al., 1988a; Godkin et al., 1988c; Lifsey et al., 1989). Variation in molecular weight is due to the presence of differential N-linked glycosylation, with the 22 kDa form having a single high-mannose type oligosaccharide and the 24 kDa form having a single complex type oligosaccharide (Helmer et al., 1988). Isoelectric variants of bTP-1 likely reflect different gene products since these variants are seen in bTP-1 from cell-free translation of bTP-1 mRNA (Anthony et al., 1988). However, it is not known whether the various isomeric forms of bTP-1 have different activities. In addition, the bTP-1 complex is a member of the alpha-interferon family and shares 80% amino-acid sequence homology with oTP-1, 50% with bovine alpha-interferon class I and 72% with bovine alpha-interferon class II (Imakawa et al., 1989). Bovine TP-1 possesses antiviral activity characteristic of other interferons (Godkin et al., 1988b). Antiviral activity has been detected in bovine conceptus conditioned medium as early as Day 10-11 (Betteridge et al., 1988) and in uterine fluids at Day 15 of gestation (Short et al., 1989). Bovine TP-1 does not appear to have any LH-like activity since no activity has been detected in homogenates from Days 16-20 bovine conceptuses when tested in LH bioassay using Leydig cells (Henricks and Poffenbarger, 1984).

In-vivo infusion of bTP-1 into the uterine lumen of cyclic cows from Days 14 to 17 extends CL lifespan by inhibiting endometrial release of $PGF_{2\alpha}$ into the posterior vena cava (Helmer et al., 1989b). In vitro studies support the hypothesis that bTP-1 blocks $PGF_{2\alpha}$ synthesis. Bovine TP-1, like bCSP, inhibits PGF secretion from endometrial explants collected at Day 17 of the estrous cycle and induces the synthesis of EPSI proteins (Helmer et al., 1989a;

Gross et al., 1988a). However, bTP-1, unlike bCSP, increases endometrial secretion of PGE_2 (Helmer et al., 1989a; Gross et al., 1988a), suggesting that other components of bCSP may block the effects of bTP-1 on PGE_2 secretion.

It is now hypothesized that bTP-1 alters luteal lifespan through an antiluteolytic-anti-PGF mechanism with the induction of endometrial EPSI proteins (Helmer et al., 1989a). Because bTP-1 inhibits only PGF while increasing PGE_2 in in-vitro studies, contrary to inhibition of $PGF_{2\alpha}$ and PGE_2 by EPSI proteins, one must speculate that bTP-1 may only act upon the endometrial epithelium in the induction of EPSI proteins. This assessment is based upon results of the work of Fortier et al. (1988) indicating that endometrial epithelial cells and stromal cells were cultured separately and shown to predominantly secrete $PGF_{2\alpha}$ and PGE_2, respectively. Thus, bTP-1 may act on the endometrium and decrease PGF production through the induction of EPSI proteins and act on the stromal cells through a different mechanism and increase PGE_2 secretion.

Putney et al. (1988b) quantified bTP-1 production in conditioned medium from conceptuses cultured in vitro under elevated temperature and at 39°C. They demonstrated that Day 17 conceptuses exposed to elevated temperature secrete 72% less bTP-1 than conceptuses cultured at 39°C. Contrary to the previous study, Geisert et al. (1988b) did not detect a qualitative decrease but an increase in the in-vitro production of bTP-1 from conceptuses recovered from severely heat-stressed cows. However, their observation on bTP-1 production was based on gross evaluation of fluorographs after loading similar counts of radiolabelled conditioned medium. It is difficult to estimate the total production of bTP-1 per culture under these conditions. Nonetheless, they noticed that heat-stressed embryos were smaller than control conceptuses. Since Geisert et al. (1988a) reported

that elongated conceptuses secrete more bTP-1 than small conceptuses, it is possible that a retarded heat-stressed conceptus may not secrete sufficient bTP-1 to extend CL lifespan.

Ovine trophoblast protein-1. The antiluteolytic component in ovine conceptus homogenates was first named "trophoblastin" by Martal et al. (1979). This active component was then characterized and referred to as "protein X" (Godkin et al., 1982) and later as "ovine trophoblast protein-1" (oTP-1) since this protein was the first major secreted protein from conceptus trophectoderm (Godkin et al., 1984a). Ovine TP-1 has an apparent molecular weight of 19 kDa, consists of at least 3-4 isoelectric forms (5.3 to 5.7 range) (Godkin et al, 1982, 1984a; Hansen et al., 1985; Ashworth et al., 1989) and is not glycosylated (Anthony et al., 1988). Ovine TP-1 is a member of the alpha-interferon family (Imakawa et al., 1987, 1989; Stewart et al., 1987, 1989b; Charpigny et al., 1988), possesses antiviral activity (Pontzer et al., 1988; Roberts et al., 1989) and immunosupressive activity (Newton et al., 1989b; Niwano et al., 1989) characteristic of other interferon molecules.

Specific oTP-1 receptors, with a high and low-affinity component, are present in endometrial tissues of cyclic and pregnant ewes (Godkin et al., 1984a; Knickerbocker and Niswender, 1989; Hansen et al., 1989). On Days 8 and 12, unoccupied oTP-1 receptors are similar between cyclic and pregnant ewes, but diminished following Day 12 in pregnant ewes (Knickerbocker and Niswender, 1989).

During pregnancy, secretion of oTP-1 is detectable between Days 10-21 in conditioned medium (Godkin et al., 1982; Hansen et al., 1985; Ashworth and Bazer, 1989) and between Days 14-24, but not at Day 12, in uterine fluids (Kazemi et al., 1988). The secretion rate of oTP-1 increases as the conceptus elongates (Nephew et al., 1989). Furthermore, Hansen et al. (1988) did not

detect any mRNA for oTP-1 in Day 12 ovine conceptuses but the concentration for mRNA was the highest in Day 14 conceptuses and decreased 200-fold in Day 22 conceptuses. Recently, Ott et al. (1989) discovered that the chorioallantois secretes immunoreactive oTP-1 between Days 25 and 45 of gestation.

Intrauterine infusion of oTP-1 into the uterine lumen of cyclic ewes from Day 12 to 20 prolongs the CL lifespan for about 3-4 days (Godkin et al., 1984b). Contrary to intrauterine infusion of oCSP from which oTP-1 has been removed, infusion of oTP-1 into the uterine lumen on Days 12-14.5 of the cycle extends CL lifespan and inhibits uterine $PGF_{2\alpha}$ response to an estradiol (Day 14) and oxytocin (Day 15) challenge (Vallet et al., 1988). As explained previously, during luteolysis, oxytocin stimulates uterine secretion of $PGF_{2\alpha}$ through activation of the inositol phosphate second messenger (IP) (Flint et al., 1986; Mirando et al., 1990; Vallet et al., 1989). Vallet et al. (1989), using a perifusion system, demonstrated that ewes treated in utero with oTP-1 on Days 12 to 14 of the cycle have decreased in-vitro secretion of PGF in response to oxytocin. In a similar experimental design, Mirando et al. (1990) reported that on Day 16 of pregnancy (but not Day 12) daily intrauterine infusion of oCSP (containing 100 μg oTP-1/day) inhibited endometrial oxytocin-stimulation of IP turnover. Since 1) endometrial concentrations of oxytocin receptors are lower during pregnancy than during the estrous cycle (McCracken et al., 1984b; Flint et al., 1986), 2) oTP-1 seems to have no access to the maternal vasculature and 3) oTP-1 does not stimulate progesterone production by luteal cells (Godkin et al., 1984a), it can be postulated that oTP-1 suppresses the luteolytic process by decreasing the number of oxytocin receptors on endometrial membranes to block oxytocin-induced pulsatile release of $PGF_{2\alpha}$.

By suppressing uterine $PGF_{2\alpha}$ secretion, these results from sheep and cattle suggest that bTP-1 and oTP-1 are the only conceptus proteins necessary for luteal maintenance.

Other Conceptus Signals Altering Luteal Function

Pregnant ewes are more resistant to the administration of luteolytic doses of $PGF_{2\alpha}$ than cyclic ewes (Silvia et al., 1984; Nephew et al., 1989; Pratt et al., 1977; Nancarrow et al., 1982). For example, intramuscular administration of 100 µg synthetic $PGF_{2\alpha}$ induced luteolysis in only 8 of 23 Day 21 pregnant ewes while the same dose induced luteolysis in 43 of 46 Day 10-11 cyclic ewes (Nancarrow et al., 1982). In addition, the resistance of pregnant ewes to luteolysis is associated with the amount of conceptus tissue rather than the number of CL present (Silvia et al., 1984; Nephew et al., 1989; Nancarrow et al., 1982). Silvia et al. (1986), by treating pregnant ewes with $PGF_{2\alpha}$ on Days 10, 13, 16, 19, 22, 26 and 30 post estrus, discovered that progesterone concentrations decreased by 24 h post-treatment in all groups, but returned to pre-treatment levels only in pregnant ewes on Days 13 and 16, with partial luteal recovery on Days 19 and 22. They concluded that the pregnant CL is more resistant to a luteolytic dose of $PGF_{2\alpha}$ at approximately Day 13 to 19 of gestation.

Until now, no explanation has been given for the early increase in peripheral concentrations of progesterone in pregnant cows (Henricks and Dickey, 1970; Ford et al., 1979a; Lukaszewska and Hansel, 1980; Hansel, 1981; Lamming et al., 1989; Schallenberger et al., 1989). Conditioned medium from Day 14 bovine trophoblastic vesicles (trophectoderm and endoderm) stimulates progesterone secretion by rat granulosa cells, indicating that bovine conceptuses may secrete luteotrophic substances (Plante et al., 1987).

Beal et al. (1981) discovered that homogenates and extracts of homogenates of Day 18 bovine conceptuses contain small molecules (< 12 kDa) with luteotrophic activity towards dispersed bovine luteal cells. These luteotrophic molecules were further characterized by Hickey and Hansel (1987). They are secreted in vitro by Days 13-18 bovine conceptuses as small (< 10 kDa), lipid-soluble, heat-labile luteotrophic molecules and may be a steroid, prostanoid or a conceptus derived platelet activating factor (PAF). Ovine conceptus and ovine conceptus homogenates from Days 13-15 pregnant ewes also stimulate progesterone production in an in-vitro system using ovine or bovine luteal cells (Godkin et al., 1978).

These results cannot be explained by the antiluteolytic-antiPGF activity of bTP-1 and oTP-1. The conceptus must secrete or induce endometrial secretion of compounds with antiluteolytic-antiprotective and/or luteotrophic activity. The leading candidates are PGE_2 and platelet activating factors (PAF). There is also evidence for a potential role for PGI_2 in luteal function.

Platelet activating factors. The finding that PAF-like molecules play critical roles in implantation and in the establishment of pregnancy in rodents and in humans raised the question of its presence and role in other species (O'Neill, 1985a, 1985b, 1985c; O'Neill et al., 1985a, 1985b). In mice, the embryo secretes PAF-like compounds as early as Day 1, inducing a significant thrombocytopenia from Day 1 to Day 6 of gestation (O'Neill, 1985a). Similar results have been found in the human (O'Neill et al., 1985a, 1985b; O'Neill, 1987). Platelet activating factor is a small molecule with biochemical features similar to the low molecular weight luteotrophic compounds reported by Hickey and Hansel (1987). Platelet activating factor has been shown to increase endometrial cell secretion of PGE_2 in humans (Smith and Kelly,

1988) and to have luteotrophic activity in human granulosa cell cultures (O'Neill, 1987), but not in bovine luteal cell cultures (Hansel et al., 1989). Since PAF did not exhibit any luteotrophic activity in the presence of bovine luteal cells, PAF and the luteotrophic compound reported by Hickey and Hansel (1987) are probably different molecules. Nonetheless, bovine conceptuses may secrete PAF-like compounds from Days 7 to 16 of gestation because platelet counts decrease during this period (Hansel et al., 1989), but not earlier (Thibodeaux et al., 1990). Thus, if PAF-like molecules are secreted by early bovine and ovine embryos, these molecules may not act directly on the CL as luteotrophic compounds but may act indirectly by increasing endometrial synthesis of PGE_2, which is a potent luteotrophic molecule (see below).

Activated platelets release compounds, such as serotonin and platelet-derived growth factor, that stimulate progesterone synthesis by bovine luteal cells (Hansel et al., 1989). Hansel et al. (1989) hypothesized that activation of platelets during early gestation, under the influence of PAF-like molecules or other embryonic compounds, releases potent luteotrophic molecules which stimulate progesterone synthesis by the CL.

Prostaglandin E_2. Early conceptuses secrete prostaglandins as early as Day 12-13 of gestation in cattle (Shemesh et al., 1979; Lewis et al., 1982) and in sheep (Marcus, 1981; Hyland et al., 1982; Lewis and Waterman, 1985). However, only two of the tested prostaglandins (PGE_2 and PGI_2) have been shown to have luteotrophic activity in vitro and in vivo.

Around the expected time of luteolysis in the sheep, PGE_2 concentrations are greatly increased in the utero-ovarian vessels of pregnant ewes as compared to cyclic ewes (Ellinwood et al., 1979a; Silvia et al., 1981). This increase may be of embryonic (Marcus, 1981; Hyland et al., 1982; Lewis

and Waterman, 1985) and endometrial origin since endometrium from Day 13-15 pregnant ewes produce higher amounts of PGE_2 (Lewis et al., 1978; Lacroix and Kann, 1982; Ellinwood et al., 1979a). Prostaglandin E_2 has been shown to be a potent luteotrophic compound when tested with luteal cells in culture (Silvia et al., 1984; Sellner and Wickersham, 1970; Speroff and Ramwell, 1970; Fitz et al., 1984a, 1984b) and a potent vasodilator that increases utero-ovarian blood flow (Bergstrom et al., 1968; Thatcher et al., 1984b). In addition, CL from Day 13-14 cyclic and pregnant ewes have a larger number of large luteal cells than at any other time of the cycle (Fitz and Sawyer, 1982). This is an important feature since large luteal cells contain most of the receptors for $PGF_{2\alpha}$ and PGE_2 (Fitz et al., 1982). Thus, this increase in PGE_2 secretion may play a significant role in CL maintenance in early pregnancy. In-vivo experiments support this concept. In cyclic ewes, administration of PGE_2 into the horn ipsilateral to the CL, but not contralateral to the CL, delays luteal regression by two days (Pratt et al., 1977; Magness et al., 1981). Concurrent administration of PGE_2 and $PGF_{2\alpha}$ blocks luteolytic action of $PGF_{2\alpha}$ (Henderson et al., 1977; Mapletoft et al., 1977; Reynolds et al., 1981).

Endometrial tissues from pregnant cows and bCSP-treated endometrial tissues from cyclic cows do not secrete higher levels of PGE_2 in vitro than that from control cyclic cows (Curl et al., 1983; Gross et al., 1988a, 1988c). Only bTP-1 has been reported to increase endometrial production of PGE_2 in vitro (Helmer et al., 1989a). These results suggest that the bovine conceptus may be the only source for an increase in PGE_2 during early pregnancy in cattle (Shemesh et al., 1979; Lewis et al., 1982). In addition, results from in-vivo studies in cattle are not as conclusive about the biological activity of PGE_2 as in the sheep. In one study, intrauterine infusion of PGE_2 extended the estrous cycle (Gimenez and Henricks, 1983) whereas in another, PGE_2 alone

had no effect (Reynolds et al., 1983). Intrauterine administration of PGE_2 did not affect PGFM stimulation in response to injections of estradiol or $PGF_{2\alpha}$ (Chenault et al., 1984). However, Reynolds et al. (1983) showed that cows receiving intrauterine infusion of PGE_2 and estradiol from Day 13 to 21 had prolonged estrous cycles (> 21 days) compared to cows receiving vehicle, estradiol or PGE_2 (< 21 days). They suggested that PGE_2 acts synergistically with estradiol to directly or indirectly extend CL lifespan. Since estradiol is secreted in small amounts by early bovine embryos (Gadsby et al., 1980; Eley et al., 1983), this may be biologically feasible.

Prostacyclins. Prostacyclin (PGI_2) is a potent vasodilator (Johnson et al., 1976), which stimulates progesterone synthesis of bovine luteal cells in culture (Fitz et al., 1984a; Milvae and Hansel, 1980b) and is secreted in high concentrations by endometrial cultures of Day 12-15 pregnant ewes (Marcus, 1981). Milvae and Hansel (1980b), by injecting PGI_2 into the bovine CL, observed an increase in peripheral concentrations of progesterone within 5 min that persisted for at least 7 h. Marcus (1981) suggested that PGI_2 may be acting alone or in concert with PGE2 (and possibly other compounds) to alter luteal functions.

Alpha-Interferons and their Role in Reproduction

Biochemical Characteristics of Alpha-Interferons and their Receptors

Interferons were first described as a group of proteins produced in response to a viral infection, which subsequently induced cell resistance to a related or unrelated virus (Isaacs and Lindenman, 1957). The interferons

(IFN[1]) were classified into three groups on the basis of cellular origin and biochemical properties (Stewart, 1980). Leukocytes and fibroblasts after exposure to virus, doubled-stranded RNA or other inducers produced mainly IFN-α and IFN-β, respectively, which are usually acid-stable proteins (Friedman, 1981). On the other hand, T lymphocytes after stimulation with mitogens or specific antigens produced mainly IFN-γ, an acid-labile protein (Friedman, 1981; Trinchieri and Perussia, 1985). Today, this classification has become blurred with the study of more cell types, but it is still clear with gene sequencing that at least three families (α, β and γ) of IFN exist (Vilcek, 1984). There is high homology between IFN-α and IFN-β genes indicating a common ancestral gene (Pestka et al., 1987), while IFN-γ differs greatly from IFN-α and IFN-β (Pestka et al., 1987).

In cattle and humans, there is a large family of distinct IFN genes (Capon et al., 1985). In particular, genes coding for IFN-α can be divided into two distinct classes. Class I genes code for 165-166 amino acid proteins whereas class II code for 172 amino acid polypeptides. The bovine IFN-α, class I sub-family (BoIFN-α_I) contains approximately 10-12 subtypes whereas bovine IFN-α, class II (BoIFN-α_{II}) contains 15-20 members (Capon et al., 1985). Recently, bTP-1 and oTP-1 have been shown to be members of the IFN-α_{II} sub-family (Imakawa et al., 1987, 1989; Stewart et al., 1987, 1989b; Charpigny et al., 1988). Bovine TP-1 and oTP-1 contain 172 amino acids and share around 70% amino acid sequence homology with BoIFN-α_{II} (Imakawa et al., 1989).

Several HuIFN-α molecules are O-glycosylated, but only one species of HuIFN-α has been shown to contain the sequence (Asn-X-Ser/Thr) necessary

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[1] The abbreviation IFN refers in the present chapter to the three classes (α, β and γ) of interferons, unless followed by a Greek letter indicating a specific class. In the next chapters, IFN will refer uniquely to alpha interferons.

for N-glycosylation (Pestka et al., 1987). In the bovine, all of the known BoIFN-α genes, except bTP-1 (Asn-78) lack the N-glycosylation site (Imakawa et al., 1989). This amino acid sequence is not present in oTP-1 (Imakawa et al., 1989).

Interferons exert their effects through cell-surface specific receptors having high and low affinity components (Pestka et al., 1987), though the functional significance of low affinity binding sites is controversial (see Pestka et al., 1987). Alpha- and beta-interferons, but not IFN-γ, share and compete for similar receptors due to their close amino acid sequence homology (IFN-α/β, type I receptors; IFN-γ, type II receptors) (Pestka et al., 1987; Branca and Baglioni, 1981; Rubinstein and Orchansky, 1986). The different subtypes of IFN-α may express a slightly different biological activity depending on their particular specificities for receptors (Pestka et al., 1987; Branca and Baglioni, 1981; Rubinstein and Orchansky, 1986).

Receptor binding analysis has further supported the similarities between oTP-1 and IFN-α. Binding of HuIFN-α or recombinant BoIFN-α (rBoIFN-α) to membrane receptors from uteri of cyclic ewes is inhibited by purified oTP-1 (Hansen et al., 1989; Stewart et al., 1987). There is, however, evidence for differential binding of oTP-1 and rBoIFN-α to ovine endometrial receptors, with oTP-1 binding to a high-affinity and low-affinity receptor and rBoIFN-α binding only to the high-affinity receptor (Hansen et al., 1989).

Potential Role for Alpha-Interferons in Reproductive Biology

Alpha-interferons have been detected in amnionic fluid, fetal tissue, placenta and decidua of humans (Lebon et al., 1982; Weislow et al., 1983; Bocci et al., 1985; Chard et al., 1986), and in murine placentae (Fowler et al., 1980). However, only speculative functions have been attributed to these molecules

during pregnancy. Bovine TP-1 and oTP-1 are the only two native IFN-α molecules with known functions in early gestation. Nonetheless, the finding that bTP-1 and oTP-1 are IFN-α molecules has broadened the potential roles of IFN-α molecules to include reproduction. The following will be a brief description of the mechanism of action of IFN-α on target cells and a description of the biological functions attributed to IFN-α, of which some may prove critical to the role played by IFN-α in the establishment and maintenance of pregnancy in cattle and sheep.

Mechanisms of action. Though there are no unequivocal explanations available as yet for the mechanisms of action of IFN-α, a few documented biochemical changes may partially explain some of these actions. Interferon-treatment of cells induces many enzymes, of which two are well-known for their inhibitory action on translation (Pestka et al., 1987; Bielefeldt Ohmann et al., 1987; Nielsen-Hamilton, 1989). One synthesized-enzyme is a 2'5'-oligo-adenylate synthetase whose polyadenylated products stimulates an endoribonuclease (RNAase F). The ribonuclease cleaves mRNA and rRNA. The other enzyme is the activated protein kinase which phosphorylates and inactivates the alpha-subunit of the translation initiation factor (EIF-2), thus inhibiting protein synthesis (Pestka et al., 1987; Bielefeldt Ohmann et al., 1987; Nielsen-Hamilton, 1989). Alpha-interferons have also been reported to decrease or increase components of the cell surface (Rubinstein and Orchansky, 1986; Kirchner, 1984; Faltynek et al., 1984). In one study, incubation of lymphoblastoid cells for 3 days with IFN-α diminished by 50% the number of insulin receptors, but not the number of transferrin receptors (Faltynek et al., 1984). Human IFN-α can increase the amount of class 1 human histocompatibility antigens in most human cell lines (Kirchner, 1984).

Inhibition of prostaglandin synthesis. It is likely that non-conceptus IFN-α can simulate the antiluteolytic effect of bTP-1 and oTP-1 when given to cyclic animals. Human IFN-α inhibits secretion of $PGF_{2\alpha}$ by ovine endometrial cells (Salamonsen et al., 1988, 1989). In sheep, continuous intrauterine infusion of 2 mg/day rBoIFN-α from Day 9 to 19 extends luteal lifespan (until at least 19 days) and decreases circulating levels of PGFM (Stewart et al., 1989a). However, there is no extension of the estrous cycle length for cyclic ewes receiving twice daily administrations of either 50 μg rHuIFN-α intramuscularly (Davis and Ott, 1989) or 2 mg rBoIFN-α in utero (Schalue-Francis et al., 1989). Intrauterine infusion of only 25 μg oTP-1 is required to extend the interestrous interval to 27 days (Schalue-Francis et al., 1989). These results suggest that non-conceptus IFN-α are less potent antiluteolytic molecules than oTP-1, which may be due to their differential ability to bind to oTP-1 receptors (Hansen et al., 1989).

Non-embryonic IFN-α can also suppress prostaglandin synthesis in other tissues. PGE_2 synthesis was reduced when human mononuclear leucocytes (Dore-Duffy et al., 1983) or human peripheral monocytes (Browning and Ribolini, 1987) were preincubated with IFN-α. Alpha-interferon decreases prostaglandin formation from mouse macrophages by diminishing liberation of arachidonic acid from phospholipids (Boraschi et al., 1985).

Antiviral activity. There is no single mechanism accepted for the inhibition of viral replication. The degree of antiviral activity observed with an IFN-α molecule is dependent upon the cell type and group of virus (Bielefeldt Ohmann et al., 1987; Taylor-Papadimitriou, 1984). At least two pathways are involved in the antiviral activity. They are the 2'5'-oligo-adenylate synthetase and the protein kinase which are known to inhibit

translation of viral message (Bielefeldt Ohmann et al., 1987; Taylor-Papadimitriou, 1984). The bovine, human and mouse IFN-α molecules contain about 10^8 units/mg of antiviral activity (Pontzer et al., 1988; Roberts et al., 1989) where one antiviral unit results in 50% inhibition of cell monolayer lysis (Familleti et al., 1981). This antiviral activity can help in the control of growth of pathogenic microorganisms that can be detrimental to the survival of the conceptus.

Antiproliferative activity. Alpha-interferons are well known to inhibit the proliferation of many cell types (Taylor-Papadimitriou and Rozengurt, 1985). Alpha-interferons by inhibiting translation and synthesis of proteins may extend the length of the cell cycle since proteins are required for the progression from G1 to S. There is evidence that within a developing embryo there are different centers of proliferation and differentiation (Dickson, 1966; Denker. 1983). Thus, the secretion of a molecule with antiproliferative activity may be essential for its development.

Immunosuppressive activity. Interferon molecules regulate many functions of the immune system but most of them are induced by IFN-γ (Rubinstein and Orchansky, 1986). Alpha-interferons inhibit the primary antibody response (Vignaux et al., 1980), augment natural killer cell activity and suppress incorporation of [^{3}H]thymidine into mitogen-stimulated lymphocytes (Roberts et al., 1989; Niwano et al., 1989). Since the conceptus is an allograft containing paternal antigens (Clarke et al., 1980), local suppression of the immune system may be important in establishment of gestation.

Maintenance of Luteal Function by Exogenous Agents and its Effect on Fertility

After a single natural or artificial insemination, the fertilization rate for both dairy and beef cows is usually between 89 and 100% (Diskin and Sreenan, 1980; Roche et al., 1981), while only 50-60% of bred cows give birth to an offspring (Sreenan, 1981). Collection of zygote, embryo and fetus at various stages after breeding indicates that early embryonic death accounts for 75-80% of the wastage; with the greatest lost occurring around Day 8-18 (Diskin and Sreenan, 1980; Roche et al., 1981). During that Day 8-18 period, it is difficult to know whether luteolysis is the cause of the death of the embryo, or if the embryo dies due to its inability to signal its presence to the mother. In either case, the resulting decrease in progesterone, if prevented, may improve the fertility rate. This hypothesis has been tested by the administration of progesterone or presumed luteotrophic agents.

Administration of Progesterone

In most studies, cows receiving exogenous progesterone tend to have higher pregnancy rates (ranging from 10-60%), but the control cows in those studies usually had a very low pregnancy rate (16-42%) (see Sreenan and Diskin, 1983). Sreenan and Diskin (1983) reported two experiments in beef heifers and a third one in dairy cows in which administration of progesterone from Days 10 to 20 increased the pregnancy rate by 17% and 27% in beef cattle and by 2% in dairy cows, respectively. However, these increased pregnancy rates were not statistically different from control cows with pregnancy rates ranging from 45-52% for beef and 65% for dairy cows.

Administration of Luteotrophic Substances

Several substances have luteotrophic activity but only human chorionic gonadotropin (hCG), LH, and GnRH (GnRH and its analogues) will be discussed in this section. The administration of these three hormones to cyclic cows extends the interestrous interval (Wiltbank et al., 1961; Donaldson and Hansel, 1965b; Seguin et al., 1977; Macmillan et al., 1985a), possibly by a direct action on the CL and luteinization of follicles (Sreenan and Diskin, 1983; McDermott et al., 1986; Macmillan et al., 1985b). Injection of hCG to bred cows tends to increase the pregnancy rate, but not significantly. Thatcher et al. (1987) found that administration of hCG at Day 15 of gestation, significantly improved the subsequent fertility by 7% on one farm and 1% on a second farm. However, control cows had a very low pregnancy rate (10-15%). An increase of 11.5% in pregnancy rate was obtained with one injection of 10 μg GnRH-analogue at Day 11-13 post breeding (Macmillan et al., 1986). Heyman et al. (1987) reported a 17% increase in pregnancy rate when a cryopreserved bovine embryos were cotransfered with two trophoblastic vesicles. The trophoblastic vesicles may have increased the total production of bTP-1.

CHAPTER 3
PURIFICATION OF bTP-1 COMPLEX AND QUANTIFICATION OF ISOMERIC FORMS OF bTP-1 AS AFFECTED BY CULTURE CONDITIONS

Introduction

In cattle, maintenance of early pregnancy depends upon the ability of a Day 16-18 conceptus to synthesize biochemical messages which will extend the functional lifespan of the CL. Experiments utilizing embryo removal (Northey and French, 1980; Humblot and Dalla Porta, 1984; Albihn, 1988), embryo transfer (Betteridge et al., 1980, 1984) and intrauterine infusion of Day 16-18 conceptus homogenates or secretions to cyclic heifers (Northey and French, 1980; Humblot and Dalla Porta, 1984; Knickerbocker et al., 1986b), demonstrated the critical role played by Day 16-18 conceptuses in the extension of the CL lifespan in the cow. The early bovine conceptus is engaged in the secretion of prostaglandins (Shemesh et al., 1979; Lewis et al., 1982), steroids (Gadsby et al., 1980; Eley et al., 1983) and polypeptides (Bartol et al., 1985a). Of these, the bTP-1 polypeptide complex seems to be a key component in prolonging luteal lifespan by inhibiting endometrial synthesis of prostaglandin $F_{2\alpha}$ (Helmer et al., 1989a, 1989b). The bTP-1 complex is a group of proteins originally defined as low-molecular-weight acidic proteins that cross-react immunologically with antiserum to oTP-1 (Helmer et al., 1987). Although in situ hybridization using oTP-1 cDNA probes detected bTP-1 mRNAs in bovine conceptus trophectoderm as early as Day 12 (Farin et al.,

1989), bTP-1 is secreted in greater quantities between Days 17-22 of gestation (Godkin et al., 1988c). Both oTP-1 and bTP-1 have been classified recently as members of the interferon-α_{II} family (Imakawa et al., 1987, 1989; Stewart et al., 1987, 1989b; Charpigny et al., 1988).

The bTP-1 complex consists of at least 7-9 isomers divided in two predominant molecular weight classes (22 kDa and 24 kDa), with isoelectric point values of 6.8-5.6 (Bartol et al., 1985a; Helmer et al., 1987, 1989b; Anthony et al., 1988; Geisert et al., 1988a; Godkin et al., 1988c; Lifsey et al., 1989). Variation in molecular weight is due to differential glycosylation; the 22 kDa form having a single high-mannose oligosaccharide chain and the 24 kDa form having a single complex type oligosaccharide (Helmer et al., 1988). Isoelectric variants of bTP-1 likely reflect different gene products since they are also present when bTP-1 is formed from cell-free translation of bTP-1 mRNA (Anthony et al., 1988). However, it is not known whether the various isomeric forms of bTP-1 have different activities.

Geisert et al. (1988a), using fluorography on [^{3}H]labelled conditioned medium from Day 15-17 bovine conceptuses, could faintly detect two bTP-1 isomers from conceptuses measuring 17 mm, while 2, 8 and 11 isomeric forms were detectable from conceptuses measuring 40, 100 and greater than 100 mm, respectively. Similar studies (Godkin et al., 1988c) performed on Day 17-38 conceptuses demonstrated that the complexity of bTP-1 increased from at least five isomers for Day 17 conceptuses to a maximum of at least 10 isomers for Day 22 conceptuses. After Day 22, the heterogeneity of the complex configuration decreased and only three isomers were present at Day 38. The length of time conceptuses are kept in culture seems to influence the isomeric forms of secreted bTP-1. Lifsey et al. (1989) cultured Day 18-20 conceptuses and detected at least seven isomeric forms of bTP-1 throughout

Days 1 and 2 of culture, but only three isomeric forms on Day 3 of culture. In addition, Helmer et al. (1989b) purified at least 10 isomeric forms of bTP-1, including 3 at 22 kDa, 5 at 24 kDa and 2 at 26 kDa. In another study, only three isomeric forms, in the 21 kDa range, were purified (Lifsey et al., 1989).

The bTP-1 complex is present in uterine fluids of pregnant cows (Kazemi et al., 1988). However, at present, bTP-1 only has been purified from conceptus-conditioned medium. In-vitro culture conditions are likely to affect yields of bTP-1. Superovulation could be a method for increasing the yield of conceptus tissue for bTP-1 production. In the present study, production of bTP-1 by Day 17-18 bovine conceptuses cultured in vitro for a period of 3 to 4 days was characterized. Of particular interest was quantification of production of individual isomers of bTP-1, and evaluation of how relative amounts of each isomer was affected by culture conditions. Additionally, a purification scheme for bTP-1 from conditioned medium was developed utilizing a combination of ultrafiltration and HPLC techniques.

Materials and Methods

Materials

Preparation of a modified essential medium (MEM, Gibco custom formula # 86-5007) and supplies for tissue culture were as described by Malayer et al. (1988). L-[^{35}S]methionine [Specific activity (SA)=~1300-1400 Ci/mmol), DL-[4,5-^{3}H]leucine (SA=26 Ci/mmol) and ^{125}I (SA=~169 mCi/μg) were from Amersham Corp. (Arlington Heights, IL). Supplies for electrophoresis were from vendors as follows: acrylamide, urea, dithiothreitol, and sodium dodecyl sulfate (SDS) from Research Organics

(Cleveland, OH); bis-acrylamide, gelatin, Tween-20, N,N'-diallyltartardiamide (DATD) and silver staining kit from Bio-Rad (Richmond, CA); ampholytes used in isoelectric focusing were from either Serva (Heidelberg, FRG; Servalytes) or BDH Chemicals (Poole, England; Resolytes); Coomassie Blue R-250 from Polysciences Inc. (Warrington, PA); phenylmethylsulfonyl fluoride (PMSF); Nonidet P-40 (NP-40), tris-hydroxymethyl-aminomethane (Tris), N,N,N',N'-tetramethyl-ethylenediamine (TEMED), ascorbic acid, pepstatin-A, leupeptin and sodium ethylenediamine tetraacetate (EDTA) from Sigma Chemical Co. (St. Louis, MO); sodium salicylate, 2-mercapto-ethanol, glycine and ammonium peroxydisulfate from Fisher Scientific (Orlando, FL). TSKgel G3000 PW_{XL} and TSKgel G4000 PW_{XL} filtration columns were purchased from Phenomenex (Rancho Palos Vendes, CA); Zorbax Bio-Series GF-250 gel filtration column was obtained from Dupont (Wilmington, DL); MonoQ HR 5/5 anion exchange column was from Pharmacia Inc. (Piscataway, NJ). Ovalbumin, bovine serum albumin (BSA), carbonic anhydrase and myoglobin were from Sigma Chemical Co. (St. Louis, MO). Dialysis tubing (Mr cutoff 6 000-8 000) was purchased from Spectrum Medical (Los Angeles, CA), ultrafiltration microconcentrators (Centricon-10, 30 and 100) were from Amicon (Danvers, MA) and bicinchoninic acid (BCA) was from Pierce (Rockford, IL). Dulbecco's phosphate buffered saline (D-PBS) was from Gibco (Grand Island, NY). Follicle stimulating hormone (FSH-P) was from Schering Co. (Kenilworth, NJ), 2-way-round tip Foley catheters (20 to 24 French gauge) were from American Hospital Supply (Jacksonville, FL), and Lutalyse was from Upjohn Co. (Kalamazoo, MI). Compound Hoechst 33258 (2-[2-(4-hydroxy phenyl)-6-benzimidazolyl]-6-I-methyl-4-piperazyl)-benzimidazol-3HCl) was obtained from Sigma Chemical Co. (St Louis, MO).

Characterization of bTP-1 Secretion

Exp. 1: Effect of day of culture. Five beef cows (Angus or Brangus) were observed for estrous behavior, bred by natural service, and slaughtered at Day 17-18 post-estrus. To collect conceptuses, reproductive tracts were removed within 30 min after stunning and flushed aseptically with 40 ml MEM into 100-mm petri dishes. The conceptuses were transferred to another tared petri dish containing 15 ml leucine-deficient (0.1X) MEM and weighed prior to supplementing the cultures with 100 μCi [^{3}H]leucine. Cultures were maintained for 96 h at 37°C on a rocking platform under an atmosphere of 50:47.5:2.5 (v/v/v) N_2:O_2:CO_2. Medium was collected every 24 h and replaced with fresh MEM containing [^{3}H]leucine. The conceptus-conditioned medium was centrifuged (2 000 x g; 15 min) and stored at -20°C until electrophoretic analysis.

Exp. 2: Effects of superovulation and gas exchange. Twenty cows (Holstein, Brangus or Senepol) were injected with 25 mg Lutalyse and, 3-4 days later, ten cows showing signs of estrus were assigned to the experiment. Five cows, starting on Day 8-10 of the estrous cycle, were superovulated. Briefly, FSH-P was injected intramuscularly, twice daily at 12 h apart for 4 days with doses of 5, 4, 3, and 2 mg FSH-P/injection on Days 1-4, respectively. Two injections of Lutalyse (25 mg each) were given at the time of the 5th and 6th FSH-P injections. The remaining five cows were reinjected with 25 mg Lutalyse on Day 8-12 of the cycle. These ten cows were checked three times a day for estrous behavior. At the first sign of estrus, both groups of cows were put with a fertile bull and artificially inseminated 12 h later and every 12 h until the end of estrus. Four superovulated and four non-superovulated cows were detected in heat and nonsurgically flushed at Day 17-18, using

gravity flow with infusion of D-PBS supplemented with 50 IU/ml penicillin and 50 µg/ml streptomycin through a Foley catheter (Elsden and Seidel, 1982).

Three single conceptuses were recovered from non-superovulated cows and an unknown number of conceptuses was recovered from two superovulated cows. Conceptuses from superovulated cows were usually harvested in pieces or clumps, disallowing determination of number of collected conceptuses whereas single conceptus from non-superovulated cows were recovered intact or in two pieces. Conceptuses were washed three times in 15 ml MEM before being cultured for 3 days at 38.5°C in leucine-deficient MEM supplemented with 50 µCi [^{3}H]leucine. Single conceptuses from non-superovulated cows were incubated on a rocking platform under an atmosphere of 50:45:5 (v/v/v) N_2:O_2:CO_2. Conceptus tissue from each superovulated cow was grossly divided into three dishes which were cultured on a rocking platform under 50:45:5 (v/v/v) N_2:O_2:CO_2 (trt 1), on a rocking platform under 5% CO_2-95% air (trt 2) or under 5% CO_2-95% air, but not rocked (trt 3). As described in Exp. 1, medium was changed daily and conditioned medium centrifuged (2 000 x g; 15 min) and stored at -20°C. After 72 h, cultures were stopped by separating tissue and medium during centrifugation (2 000 x g; 15 min). Tissue and medium were stored at -20°C until later analysis.

Quantification of bTP-1 secretion rates. Conditioned medium from Exp. 1 and 2 was prepared for electrophoresis by dialysis (3 changes of 4 l) against deionized water containing 0.02% (w/v) NaN_3, 0.1 mM PMSF and 1 mM EDTA. Dialysis tubing with a molecular weight exclusion limit of 6 000-8 000 kDa was used to remove low molecular weight compounds and unincorporated radiolabelled precursors. Two-dimensional (2D) sodium dodecyl sulfate, polyacrylamide gel electrophoresis (SDS-PAGE) was

performed as described by Roberts et al. (1984). Samples containing 200 000 dpm were lyophilyzed and run in the first dimension by isoelectric focusing in 4.2% (w/v) polyacrylamide tube gels containing 2.5% (v/v) pH 3-10, 1.8% (v/v) pH 5-7 and 0.8% pH 9-11 Servalytes. Tubes gels were subjected to electrophoresis in the second dimension on 12.5% polyacrylamide slab gels using the buffer system of Laemmli (1970). Gels were stained with Coomassie Blue, destained and prepared for fluorography by soaking in 1 M sodium salicylate, drying and exposing to Kodak XAR film at -70°C. Exposure times were the same for all gels. A total of 13 protein spots in the region characteristic of bTP-1 complex (Fig. 3-1) were punched using the fluorograph as template and placed directly into scintillation vials. To estimate background, two other spots were punched from each gel in unexposed regions (bottom left corner and top right corner). Pieces of acrylamide were dissolved by incubation with 500 µl 30% hydrogen peroxide at 70°C for 48 h. Prior to counting, 100 µl of 500 mM ascorbic acid was added to neutralize the hydrogen peroxide and vials were stored in the dark for 48 h. Total daily secretion of individual bTP-1 isomers was corrected mathematically for background, the total 15 ml of culture medium and either tissue weight (Exp. 1) or DNA content (Exp. 2).

DNA assay. A quantitative determination of DNA was made on conceptus tissues from Exp. 2 following Labarca and Paigen's technique (1980). Tissues were homogenized with an hand-microtome in 250 µl PBS [0.05 M Na-phosphate, pH 7.4 containing 0.02% (w/v) NaN_3 and 2 mM EDTA] and sonicated for 2 min. Aliquots of homogenates or standard (calf thymus DNA) were mixed with compound Hoechst 33258 (final concentration of 1 µg/ml) in a final volume of 1 ml. The assay was read 4-6 h later on a fluorocolorimeter

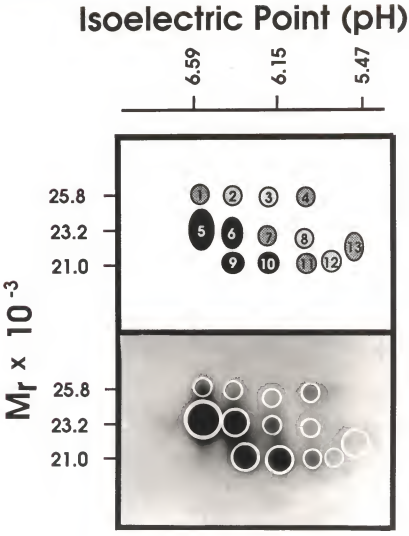


Fig. 3-1. Schematic drawing and representative fluorograph of two-dimensional gel electrophoresis of bTP-1 complex from [^{3}H]labelled conditioned medium of Day 17-18 bovine conceptuses. The 13 isoelectric variants occurring most frequently were characterized according to Mr and pI. Nine of these isomers (# 1, 2, 5, 6, 7, 8, 9, 10 and 11) are believed to be identical to 9 of 10 isomers reported to be present in purified bTP-1 and cross-reacting immunologically with anti-oTP-1 (Helmer et al., 1989b). The remaining 4 isomers have never been identified as reacting with anti-oTP-1 but, except for isomer 12, their presence in bCSP has been reported in other studies (isomer 13, Geisert et al., 1988a; isomers 3 and 4, Godkin et al., 1988c).

using an excitation wavelength of 356 nm and an emission wavelength of 458 nm.

Characterization of [^{3}H] and [^{35}S]labelled conceptus-conditioned medium by gel filtration. Two conceptuses from non-superovulated cows were cultured for three days, in 15 ml methionine-deficient (0.1X) MEM supplemented with 100 µCi [^{35}S]methionine and conditioned medium was pooled across conceptuses for each day of culture. The conceptuses were rocked under an atmosphere of 50:45:5 (v/v/v) N_2:O_2:CO_2. Medium was changed every 24 h. Aliquots of conditioned medium from these cultures and cultures with [^{3}H]leucine described previously (Exp. 2) were concentrated with a Centricon-10 microconcentrator (see below) and analyzed for elution pattern of radiolabelled proteins by gel filtration HPLC using two TSK columns assembled in tandem (TSKgel G4000 PW_{XL} followed by TSKgel G3000 PW_{XL}, each with 7.8 mm i.d. x 30 cm length). Mobile phase was 0.2 M Na-phosphate, pH 7.9 at a flow rate of 0.7 ml min. Fractions were counted for radioactivity using scintillation spectrophotometry.

Purification of bTP-1 Complex

Preparation of conditioned culture medium prior to purification. Conceptuses were cultured for 72 h in modified MEM under 50:45:5 (v/v/v) N_2:O_2:CO_2. A total of 23 Day 16-18 conceptuses from non-superovulated cows were cultured on a rocking platform, without radiolabelled leucine or methionine, for 72 h in modified MEM under 50:45:5 (v/v/v) N_2:O_2:CO_2. At the end of the culture period, freshly-harvested conditioned medium was centrifuged (2 000 x g; 15 min) and added to a bottle containing previously frozen (-20°C) conditioned medium. Conditioned medium was stored in six pools with two pools each for Days 1, 2 and 3 of culture. Only pool 1 and 2 from the first day of culture were purified in this study.

Preparation of radiolabelled conceptus proteins for quality control material. Conditioned medium from conceptus cultures performed with [^{3}H]leucine was used to generate a quality control preparation that was mixed with unlabelled medium for the purpose of monitoring conceptus proteins during purification steps. The pooled cultures were processed first with the Centricon-100 ultrafiltration device to remove high molecular weight compounds (Mr > 80-100 kDa). Two milliliters of medium was placed in each Centricon unit and centrifuged at 4 000 x g for 90 min. The filtrate was then processed with the Centricon-10 ultrafiltration device to remove low molecular weight compounds (Mr < 8-10 kDa). The resulting radiolabelled retentate, when mixed with unlabelled cultures, enabled the monitoring of Centricon membrane integrity during repeated use. Additionally, the radiolabelled profiles from chromatography steps were used to monitor resolving capabilities of the HPLC columns. Finally, degradation of the bTP-1 complex could be observed by a shift in radioactivity to the permeation volume of the HPLC gel filtration column.

Fractionation of conditioned medium using Centricon membranes. A mixture of protease inhibitors was added to non-radiolabelled conditioned medium to give a final concentration of 1 mM EDTA, 0.1 mM PMSF, 0.7 µg/l pepstatin-A, 0.5 µg/l leupeptin and 0.02 % (w/v) NaN_3. Aliquot of [^{3}H]labelled quality control material was added to the conditioned medium such that the resulting solution contained approximately 25 000 dpm/ml of radiolabelled conceptus proteins. The medium was centrifuged (10 000 x g, 4^{o}C) for 30 min and the supernatant fraction processed using Centricon ultrafiltration devices as described earlier. After each spin, the filtrate was collected and concentrated (20-30 x) using a Centricon-10 (4 000 x g for 90 min). The final retentate (~300 µl/Centricon) was then washed by centrifuging three

times with 2 ml 20 mM Tris-HCl, pH 8.0 in preparation for the HPLC anion exchange step.

After each centrifugation step, the integrity of each Centricon-10 filtrate was determined. An arbitrarily-set criteria in which 25% of radioactivity was found in the filtrate was used as an indication of membrane exhaustion. The Centricons were used in excess of 30 times and none were removed from service.

Anion exchange HPLC. Retentate from Centricon-10 was centrifuged for 3 min at 12 000 x g on a microcentrifuge before being loaded on Mono Q HR 5/5 anion exchange column (5 x 50 mm; maximum of 350 µl/injection i.e., about 9 ml conditioned medium equivalent). The HPLC system used was described by Helmer et al. (1989b). Separation was accomplished with a linear gradient of 0.0-0.48 M NaCl in 20 mM Tris-HCl, pH 8.0, with a flow rate of 1.0 ml/min. Fractions of 10 drops (~ 450 µl) were collected, counted for radioactivity and pooled as appropriate. In preliminary runs, composition of fractions was evaluated by one dimensional (1D) SDS-PAGE and fluorography. The bTP-1 peak eluted between 0.20-0.35 M NaCl and was concentrated using Centricon-10 ultrafiltration device (4 000 x g, 90 min) and washed 3 times with 0.2 M sodium phosphate buffer, pH 7.1.

Gel filtration HPLC. The concentrated bTP-1 peak prepared by anion exchange was centrifuged for 3 min at 12 000 x g and loaded on a Zorbax Bio-Series GF-250 column (9.4 mm i. d. x 250 cm length). The column was calibrated with BSA (Mr 69 kDa), ovalbumin (Mr 45 kDa), carbonic anhydrase (29 kDa) and sodium azide (salt volume). A maximum of 195 µl (about 9 ml conditioned medium equivalent) was applied per injection. Flow rate of eluent (0.2 M Na-phosphate buffer, pH 7.1) was 0.7 ml/min. Preliminary results, based on 1D SDS-PAGE, indicated that bTP-1 complex eluted at

approximately the same elution time as carbonic anhydrase (29 kDa). Fractions (3 drops/fraction; ~166 µl) corresponding to elution volumes of protein having molecular weights of ~25-35 kDa were pooled (pooled fractions 68-70 and 71-76), concentrated to a volume of ~200 µl using a Centricon-10 ultrafiltration device, washed 3 times with D-PBS supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B, and stored frozen (-20°C) in aliquots.

Verification of purity. A total of 46, 6, 11 and 34 ml of unlabelled conditioned medium from the first 24 h of culture was supplemented with radiolabelled conditioned medium and purified by batches. One run utilized a pool frozen for ~18 mo and the other runs utilized a pool frozen for less than 6 mo. At the end of each purification scheme, pooled fractions (fractions 68-70 and 71-76) from gel filtration were analyzed by 1D SDS-PAGE and silver staining. The silver staining procedure was as described in the Bio-Rad kit. The two fractions (except when one was not pure enough) were pooled and stored at -70°C.

One aliquot of dialysed conditioned culture medium (100 µg protein) and three aliquots of purified bTP-1 complex (pool of 4 batches; 7.5 µg/aliquot) were analyzed by 2D SDS-PAGE using a first dimension gel containing 6.0% (v/v) Resolytes 3.5-10.0. After electrophoresis, proteins were silver stained or transferred electrophoretically to 0.20 µm nitrocellulose paper using 200 mA current overnight at 4°C in an electroblotting chamber containing 25 mM Tris, 0.2 M glycine and 20% (v/v) methanol. The lane for molecular weight standards was cut off and stained for 5 min in 0.05% (w/v) amido black dissolved in methanol:acetic acid: deionized water (40:10:50; v:v:v). The remainder of each blot was analyzed for bTP-1 by Western blotting. After overnight incubation, at room temperature in blocking buffer [20 mM Tris-

HCl, pH 7.4 containing 1% (w/v) gelatin, 0.5 M NaCl, 0.05% (v/v) Tween 20 and 0.02% (w/v) NaN_3], blots were washed (blocking buffer without gelatin) and then incubated for 2 h at room temperature with either normal rabbit serum or anti-oTP-1 (1:100 dilution) diluted in incubation buffer [blocking buffer with 0.1% (w/v) ovalbumin instead of 1% gelatin]. Blots were washed for 1-3 h at room temperature and incubated with 1 X 10^6 cpm/ml ^{125}I-labelled-Protein-A (prepared as in Putney et al., 1988b). The radiolabelled blots were washed overnight, dried and exposed to Kodak XAR X-ray film.

Antiviral activity of purified bTP-1 complex. This assay is based on inhibition of viral cytopathic effects (Familleti et al., 1981). Briefly, monolayers of Madin-Darby bovine kidney (MDBK) cells established in 96-well microtiter plates were incubated for 16-18 h at 37°C with different sample dilutions (three-fold serial dilution). The cells were then challenged with bovine vesicular stomatitis virus and incubated for a further 24 h. Cells were fixed and stained with crystal violet to evaluate cell survival. One antiviral unit inhibits 50% of the destruction of the MDBK cell monolayer.

Protein Assay

Protein concentrations were determined by the bicinchoninic acid (BCA) protein assay (Smith et al., 1985), with BSA as standard.

Statistical Analysis

Data were analyzed via least-squares analysis of variance using the General Linear Model of the Statistical Analysis System (SAS, 1985). In all analyses, cow was considered as a random effect. Data on bTP-1 secretion were analyzed after classifying bTP-1 secretion according to each individual isomer, each molecular weight groups [sum of secretion rates for 25.8 kDa

group (isomers 1 to 4); 23.2 kDa group (isomers 5 to 8); and 21.0 kDa group (isomers 9 to 13)] and overall bTP-1 secretion (sum of secretion for all isomers). An additional analysis was performed with all isomers in the model and isomer number as a main effect. In this analysis, significant interactions between isomer number and other main effects (e.i., day, gas environment, etc.) would indicate that the relative proportion of bTP-1 isomer changed with the interacting factor.

Data on bTP-1 classified according to bTP-1 molecular weight group were analyzed such that each molecular group was analyzed separately. For all analyses, orthogonal contrasts were used to evaluate effects of day (Exp. 1: Days 1, 2 and 3 vs Day 4, Days 1 and 2 vs Day 3 and Day 1 vs Day 2; Exp. 2: Days 1 and 2 vs Day 3 and Day 1 vs Day 2). Data for Exp. 1 considered effects of cow and day. Data for Exp. 2 were analyzed in two ways. The first analysis, performed on data from three single and two superovulated conceptuses cultured under similar conditions, considered effects of treatment (single ovulation vs superovulation), cow(treatment), day and day x treatment. The second analysis, performed on data from conceptus tissues cultured under three different conditions considered effects of cow, treatment, cow x treatment, day, cow x day and treatment x day. Differences in treatment means were evaluated by orthogonal contrast (trt 1 vs trts 2 and 3, and trt 2 vs trt 3).

Results

Secretion Rate of Isomeric Forms of [^{3}H]labelled bTP-1

Fluorographs developed from 2D SDS-PAGE analysis of the bTP-1 complex within conditioned medium from Day 17-18 bovine conceptuses are presented in Figs. 3-1 and 3-2. Between four and 13 peptides were identified in the bTP-1 area, based on immunoreactivity and/or position on the gel. These isomers ranged in pI from 6.59 to 5.60 and were divided in three distinct Mr groups (25.8 $\pm$ 0.27 kDa; 23.2 $\pm$ 0.23 kDa; 21.0 $\pm$ 0.25 kDa; mean $\pm$ s.e.m.; Table 3-1). In both experiments, the most consistent and abundant isomers were 5, 6, 9 and 10. In a previous study (Helmer et al., 1989b), purified bTP-1 was shown to contain 10 isomers reacting immunologically with antiserum against oTP-1. Nine of the ten bTP-1 isomers reported by Helmer et al. (1989b) are believed to be identical to isomers 1, 2, 5, 6, 7, 8, 9 10 and 11 in the present study. The tenth isomer reported by Helmer et al. (1989b) was detected in conditioned medium from three conceptuses in the present study, and was not evaluated for its secretion rate. When present, isomer 10 was characterized by a very faint band in the 23.2 kDa range with a more basic pI than isomer 5. The remaining 4 isomers (isomers 3, 4, 12 and 13) were very faint, but detectable most of the time. None of these remaining isomers have been reported to react with anti-oTP-1. Isomer 13 appears to be present on fluorographs from Geisert et al. (1988a) and isomers 3 and 4 on fluorographs from Godkin et al. (1988c).

Effect of day of culture. In Exp. 1, the secretion rate of bTP-1 complex was affected by the culture period. The bTP-1 complex was less intense on fluorographs from conditioned medium at Day 4 of culture (Fig. 3-2) than Days 1, 2 and 3. An increased amount of high Mr proteins (Mr > 45 kDa) was

also seen on Days 3 and 4 of culture. Even though the culture period did not affect the total amount of nondialysable-[^{3}H]labelled proteins (Fig. 3-3),

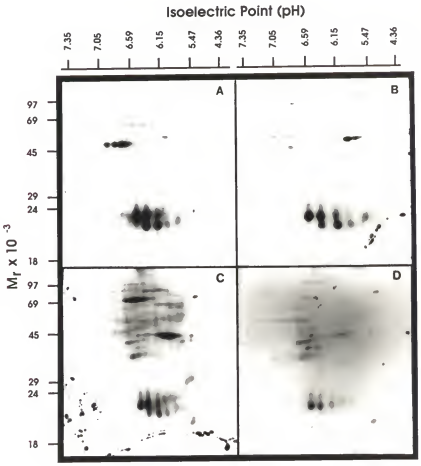


Fig. 3-2. Representative fluorographs of two-dimensional gel electrophoresis of dialysed [^{3}H]labelled conditioned medium of Day 17-18 bovine conceptuses cultured for 4 days (Days 1, 2, 3 and 4 are, respectively, in panels A, B, C and D). Equal amounts of radioactivity (200 000 dpm) were loaded on each gel.

Table 3-1. Estimate of Mr and pI of 13 peptides identified as belonging to the bTP-1 complex (n=12).

Identification number	Mr ± s.e.m. (kDa)	pI
1	25.8 ± 0.27	6.59
2	25.8 ± 0.27	6.24
3	25.8 ± 0.27	6.15
4	25.8 ± 0.27	5.80
5	23.2 ± 0.23	6.59
6	23.2 ± 0.23	6.24
7	23.2 ± 0.23	6.15
8	23.2 ± 0.23	5.80
9	21.0 ± 0.25	6.24
10	21.0 ± 0.25	6.15
11	21.0 ± 0.25	5.80
12	21.0 ± 0.25	5.70
13	21.6 ± 0.28	5.60

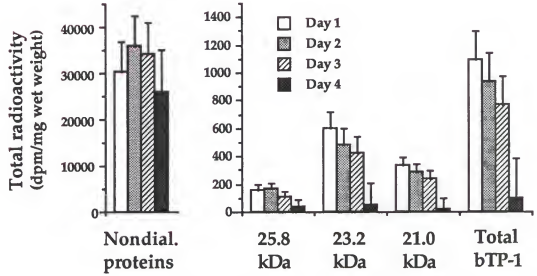


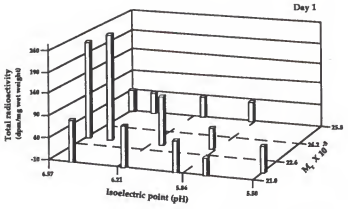
Fig. 3-3. Daily secretion of [^{3}H]labelled proteins synthesized by Day 17-18 bovine conceptuses during 4 days of in vitro culture. Columns represent least-squares means and the vertical bar, the s.e.m. Day of culture did not influence secretion rate of nondialysable proteins. Conceptuses at Day 4 of culture secreted less 25.8 kDa ($P < 0.08$), 23.2 kDa ($P < 0.03$) and 21.0 kDa ($P < 0.02$) Mr forms of bTP-1, and total bTP-1 complex ($P < 0.03$) than during the first 3 days of culture. No significant difference was detected between the first 3 days of culture.

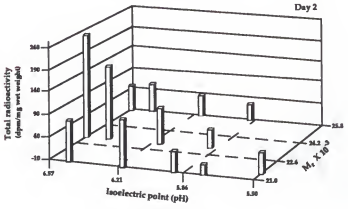
conceptuses at Day 4 of culture secreted less 25.8 kDa ($P < 0.08$), 23.2 kDa ($P < 0.03$) and 21.0 kDa ($P < 0.02$) bTP-1 Mr forms, as well as, total bTP-1 complex ($P < 0.03$) than during the first 3 days of culture. Secretion rates during the first 3 days of culture did not differ from each other. Analysis of individual bTP-1 isomers (Fig. 3-4) indicated that 8 isomers (# 1, 6, 7, 8, 10, 11 and 12, $P < 0.05$; # 9, $P < 0.06$) had a lower secretion rate at Day 4 compared to the first 3 days of culture. There was no difference in secretion of individual isomers between the first 3 days of culture, except for isomer 12 which had lower secretion at Day 2 than at Day 1 of culture. There was no isomer type x day interaction, suggesting that each of the isomers varied with day in a similar pattern.

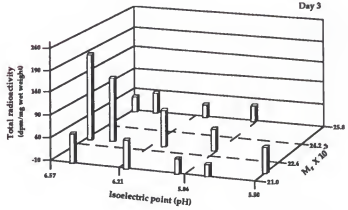
Other conceptuses, cultured in the presence of [^{35}S]methionine or [^{3}H]leucine, were analyzed by HPLC-gel filtration. Chromatogram profiles did not differ for the first 3 days of culture and Fig. 3-5 shows representative gel filtration chromatograms from Day 1 of culture. For the profile of [^{35}S]labelled proteins, the radioactive peak in the bTP-1 region (fractions 67-70) contained approximatively 41% of total recovered counts. For [^{3}H]labelled proteins, there was no peak in the bTP-1 area (fractions 63-66; approximately 6% of total recovered counts).

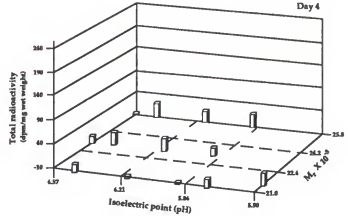
Effect of superovulation. The conceptus tissue recovered from three non-superovulated cows had total DNA content of 5.6, 7.6 and 132. 5 μg whereas total conceptus tissue harvested from two superovulated cows contained a total of 19.3 and 43.1 μg DNA. It is important to remember that conceptus tissues from each superovulated cow were grossly divided into three treatments, giving approximately 6 and 14 μg DNA per tissue culture. In the present experiment, the amount of conceptus tissue per culture was less than usual (we expected a content of > 20 μg DNA/culture), which could have affected the results. However, on a per DNA basis, single-

Fig. 3-4. Daily secretion rate of individual [^{3}H]labelled bTP-1 isomers synthesized by Day 17-18 bovine conceptuses during 4 days of in vitro culture. Isomers were identified from left to right in 3 Mr classes (25.8 kDa: isomers 1, 2, 3 and 4; 23.2 kDa: isomers 5, 6, 7 and 8; 21.0 kDa: isomers 9, 10, 11, 12 and 13). Columns represent least-squares means. Isomers 1, 6, 7, 8, 10, 11, 12 ($P < 0.05$) and 9 ($P < 0.06$) had a lower secretion rate at Day 4 compared to the first 3 days of culture. No significant difference was noticed between the first 3 days of culture, except for isomer 12 which had lower secretion at Day 2 than at Day 1. There was no isomer type x day interaction. The pooled s.e.m. for isomer 1 to 13 were 11.8, 12.2, 9.3, 9.6, 78.7, 33.7, 15.3, 7.5, 23.3, 23.9, 13.0, 5.5 and 16.8 dpm/mg wet weight, respectively.


Day 1
Total radioactivity (dpm/mg wet weight)
360
290
160
90
40
-10
6.57
6.21
5.86
5.50
Isoelectric point (pH)
25.8
24.2
22.6
21.0
$M_r \times 10^3$


Day 2
Total radioactivity (dpm/mg wet weight)
360
290
160
90
40
-10
6.57
6.21
5.86
5.50
Isoelectric point (pH)
25.8
24.2
22.6
21.0
$M_r \times 10^3$


Day 3
Total radioactivity (dpm/mg wet weight)
360
290
160
90
40
-10
6.57
6.21
5.86
5.50
Isoelectric point (pH)
25.8
24.2
22.6
21.0
$M_r \times 10^3$


Day 4
Total radioactivity (dpm/mg wet weight)
360
290
160
90
40
-10
6.57
6.21
5.86
5.50
Isoelectric point (pH)
25.8
24.2
22.6
21.0
$M_r \times 10^3$

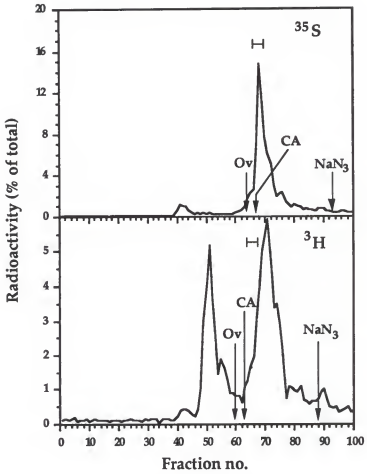


Fig. 3-5. Representative HPLC gel filtration chromatograms using two TSK columns assembled in tandem (TSKgel G4000 PW_{XL} followed by TSKgel G3000 PW_{XL}) of [^{35}S] and [^{3}H]labelled conditioned medium from Day 17-18 bovine conceptuses. Dialysed conditioned medium from the first day of culture was centrifuged (10 000 x g, 4°C) for 30 min and concentrated (20-30 x) through a Centricon-10 ultrafiltration device. Ten to 50 µl were injected over the columns (flow rate of 0.7 ml/min of 0.2 M Na-phosphate buffer, pH 7.9). Fractions were collected at an interval of either 30 sec (panel ^{35}S; 250 µl/fraction) or 32 sec (panel ^{3}H; 267 µl/fraction). Data are expressed as a percentage of the total radioactivity recovered from the columns. The horizontal line on top of both panels represents the area of bTP-1 elution as evaluated by SDS-PAGE and fluorography. Ov=ovalbumin (45 kDa), CA=carbonic anhydrase (29 kDa) and NaN_3=sodium azide (salt volume).

ovulated conceptuses secreted as much total nondialysable radioactive proteins and bTP-1 proteins as superovulated embryos (Fig. 3-6). Superovulated embryos secreted more isomer 1 ($P < 0.05$) and 9 ($P < 0.09$) than single ovulated embryos (results not shown). In this experiment, isomer 4, was secreted at a lower rate at Day 1 and 2 than at Day 3 of culture. Orthogonal contrasts for isomers 1 and 5 indicated an increase in secretion of both isomers from Day 1 to Day 2 ($P < 0.05$). Treatment, day and treatment x day did not affect type of isomer, suggesting that individual isomers were not differently affected by the in vitro culture period and superovulation. However superovulated conceptuses tended to secrete more non-dialysable proteins and more bTP-1 than the single-ovulated conceptuses.

Effect of gas exchange. There was no effect of gas atmosphere (50% N_2-45% O_2-5% CO_2, rocking platform vs 5% CO_2-95% air, with or without rocking platform) on secretion of individual bTP-1 forms, total bTP-1 or total nondialysable proteins (Fig. 3-7). Individual bTP-1 isomers were not affected by treatment and there was no treatment x isomer type or treatment x day x isomer type interaction (results not shown). Isomers 1 to 13 were secreted at similar rates at Day 1-2 as compared to Day 3 of culture. For isomers 5, 9 ,13 ($P < 0.05$) and 11 ($P < 0.09$) secretion was higher at Day 2 than Day 1. The differential changes in secretion of bTP-1 isomers with day was detected by a day x isomer type interaction ($P < 0.05$). However, conceptuses cultured on a rocking platform under 5% CO_2-95% air tended to secrete more bTP-1.

Purification of bTP-1

Ultrafiltration using Centricon-100 eliminated proteins having Mr above ~80 kDa. The ultrafiltrate from unlabelled conditioned medium spiked with [^{3}H]labelled medium was resolved by anion-exchange HPLC using a

Fig. 3-6. Daily secretion of [^{3}H]labelled proteins synthesized by Day 17-18 single or superovulated conceptuses cultured in vitro for 3 days. Columns represent least-squares means and the vertical bar, the s.e.m. Single ovulated embryos secreted as much nondialysable proteins, 25.8 kDa, 23.2 kDa, 21.0 kDa and total bTP-1 as superovulated embryos. No significant difference was detected between the 3 days of culture. Superovulated conceptuses secreted more isomer 1 ($P < 0.05$) and 9 ($P < 0.09$) than single ovulated conceptuses. There was no significant effect of isomer type x treatment, isomer type x day or isomer type x treatment x day.

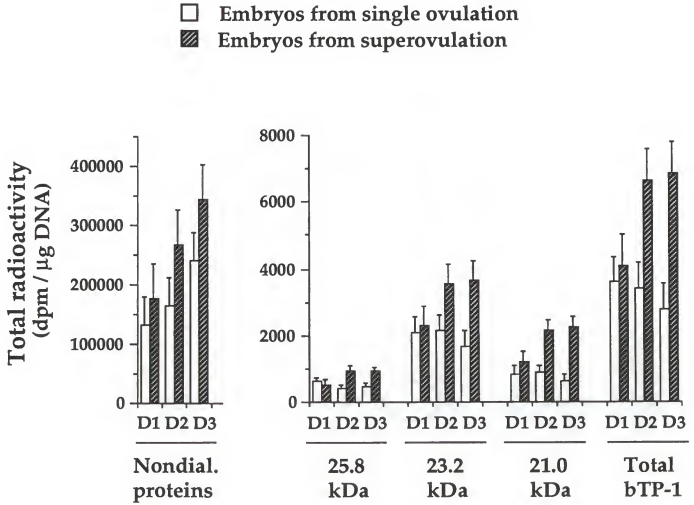

Embryos from single ovulation
Embryos from superovulation
Total radioactivity (dpm / µg DNA)
400000
300000
200000
100000
0
8000
6000
4000
2000
0
D1 D2 D3
Nondial. proteins
25.8 kDa
23.2 kDa
21.0 kDa
Total bTP-1

Fig. 3-7. Daily secretion of [^{3}H]labelled proteins synthesized by Day 17-18 conceptuses cultured in vitro for 3 days under 50% N_2-45% O_2-5% CO_2 (rocking platform), 5% CO_2-95% air (rocking platform) or 5% CO_2-95% air (no rocking platform). Columns represent least-squares means and the vertical bar, the s.e.m. There was no significant effect of culture condition, day of culture or interactions on secretion of individual bTP-1 Mr forms, total bTP-1 or total nondialysable proteins. Individual bTP-1 isomers were not affected by the treatments and there was no treatment x isomer type and treatment x day x isomer type interaction. Isomers 1 to 13 were secreted at a similar rate at Day 1-2 as compared to Day 3 of culture. For isomers 5, 9 ,13 ($P < 0.05$) and 11 ($P < 0.09$) secretion was higher at Day 2 than Day 1. There was a day x isomer type interaction ($P < 0.05$).

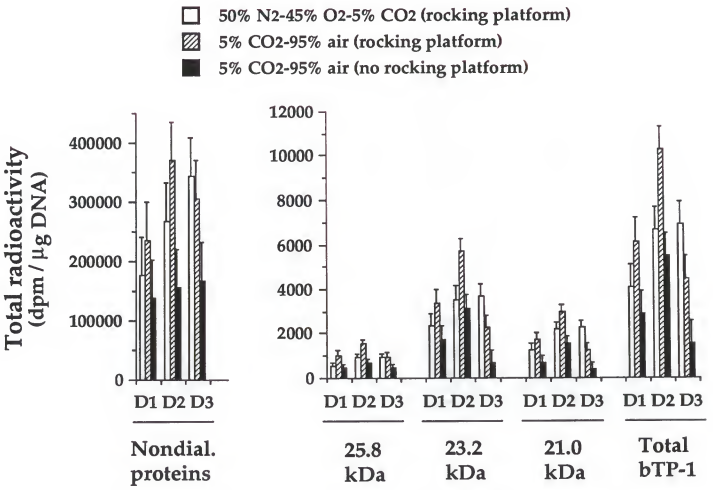
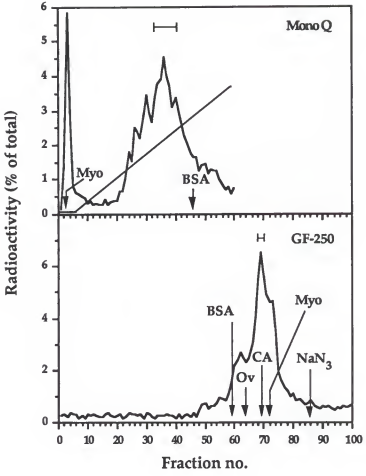

50% N2-45% O2-5% CO2 (rocking platform)
5% CO2-95% air (rocking platform)
5% CO2-95% air (no rocking platform)
Total radioactivity (dpm / µg DNA)
400000
300000
200000
100000
0
12000
10000
8000
6000
4000
2000
0
D1 D2 D3
D1 D2 D3
D1 D2 D3
D1 D2 D3
D1 D2 D3
Nondial. proteins
25.8 kDa
23.2 kDa
21.0 kDa
Total bTP-1

Fig. 3-8. Representative HPLC chromatograms of bTP-1 purification using Mono Q anion exchange and Zorbax GF-250 gel filtration columns. Unlabelled conditioned medium (34 ml) from Day 16-18 bovine conceptuses, spiked with [^{3}H]labelled medium (4-90 x 10^4 dpm/ml cold medium), was centrifuged (10 000 x g, 4°C) for 30 min, and run through a Centricon-100 ultrafiltration device. Filtrate was then concentrated using a Centricon-10 to a final volume of 1.7 ml in 20 mM Tris-HCl, pH 8.0 supplemented with 1 mM EDTA, 0.1 mM PMSF, 0.7 µg/l pepstanin-A, 0.5 µg/l leupeptin and 0.02% (w/v) NaN_3. Washed retentate was centrifuged for 3 min at 12 000 x g, and loaded on a Mono Q anion exchange column (panel Mono Q). Separation was accomplished by a linear salt gradient (0.0 to 0.48 M NaCl in 20 mM Tris-HCl, pH 8.0) at a flow rate of 1.0 ml/min (10 drops/fraction; ~450 µl). The bTP-1 peak which eluted between 0.20-0.35 M NaCl (fractions 34-42) was concentrated on Centricon-10 and washed with 0.2 M Na-phosphate buffer, pH 7.1. After 3 min of centrifugation at 12 000 x g, the Mono Q enriched bTP-1 peak was loaded over a Zorbax GF-250 column at 0.7 ml/min of 0.2 M Na-phosphate buffer, pH 7.1 (panel GF-250). Purified bTP-1 eluted in fractions 68-70 (3 drops/fraction, ~166 µl). Data are expressed as a percentage of the total radioactivity recovered from the columns. The horizontal line on top of both panels represents the area of bTP-1 elution. BSA=bovine serum albumin (69 kDa, pI ~ 6.0), Myo=myoglobin (18.5 kDa, pI ~9.6), Ov=ovalbumin (45 kDa), CA=carbonic anhydrase (29 kDa) and NaN_3=sodium azide.


Mono Q
Myo
BSA
GF-250
H
BSA
Myo
CA
Ov
NaN3
Radioactivity (% of total)
Fraction no.

Mono Q column. The chromatogram had two distinct areas (Fig. 3-8). The first area, representing highly basic proteins, was eluted with 20 mM Tris-HCl, pH 8.0. Proteins in area two (fractions 22-46) were eluted with a linear salt gradient. Gel electrophoresis indicated that bTP-1 was primarily concentrated in fractions 34-42. These fractions were concentrated and further resolved using a Zorbax Bio-Series GF-250 gel filtration column. The bTP-1 complex was recovered usually in fractions 68-76 (3 of 4 runs) or 68-70 (1 of 4 runs).

When 97 ml of conditioned medium from the first 24 h of culture collected from non-superovulated cows were processed through the purification scheme, the final purified bTP-1 pool yielded 1.29 μg protein/ml conditioned medium (19.28 μg/conceptus culture). The 2D-PAGE gel was silver stained and the purified bTP-1 pool showed six bTP-1 isomers and a faint, high Mr, acidic contaminant (45 kDa, 6.63 pI; Fig. 3-9 B). This contaminant was not an artifact of the silver staining procedure since no protein spots were detected on 2D-PAGE silver-stained gel of loading buffer alone. Immunoblotting using anti-oTP-1 confirmed the presence of bTP-1 in the purified pool (Fig. 3-9 C-D). Anti-oTP-1 detected nine bTP-1 isomers including a low Mr protein (19 kDa, pI ~ 5.5).

Purified bTP-1 from pool 1 (frozen for ~ 18 mo before purification) contained 1.14×10^5 U of antiviral activity/mg protein and pool 2 (frozen for less than 6 mo) had 1.49×10^7 U/mg protein. It is likely that storage time affects the biological activity of bTP-1 since purity, based on silver staining of 1D-PAGE gels was similar in both cases.

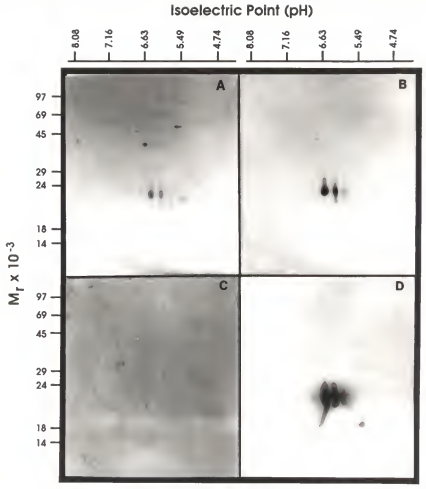


Fig. 3-9. Silver staining and immunoblotting of purified bovine trophoblast protein-1 (bTP-1). Purified bTP-1 (7.5 µg/gel) was separated by two-dimensional polyacrylamide electrophoresis and either stained with silver (B) or electrophoretically transferred to nitrocellulose paper and incubated with normal rabbit serum (C) or rabbit anti-oTP-1 antiserum (1:100 dilution, D). Dialysed Day 16-18 conceptus conditioned medium (100 µg of protein) was separated by 2D-PAGE gel and stained with silver (A).

Discussion

The secretion of the bTP-1 complex by the Day 16-18 conceptuses plays a critical role in the early recognition of pregnancy in cattle. A reliable source of bTP-1 is critical for elucidating the biological activities associated with this complex. This study demonstrated that yield of bTP-1 from pregnant cows could be increased by culturing conceptus over a prolonged period. Three days of culture did not result in a decrease in the overall secretion of the bTP-1 complex and the secretion pattern of individual bTP-1 isomers altered only slightly. In a previous study (Lifsey et al., 1989), the secretion of bTP-1 decreased significantly by the third day of incubation of Day 18-20 bovine conceptuses, as evaluated by 2D SDS-PAGE and fluorography. In the present study, a decrease in bTP-1 secretion was not detected until the fourth day of culture. Conditioned medium from the fourth day of culture was considered a poor source of bTP-1 for purification because small amounts of bTP-1 were present and because of an increase in the complexity of the proteins secreted.

There was some evidence that superovulation increased the yield of bTP-1 per cow. Total protein secreted and bTP-1 secreted had tendency to be greater with superovulated conceptuses than non-superovulated conceptuses, but these variables were not significantly different. Two of three non-superovulated cows produced less total conceptus tissue than superovulated cows. More Day 17-18 conceptuses are required to statistically test whether superovulation may increase bTP-1 yielding. In addition, present results indicate that the culture conditions may play a role in overall synthesis of bTP-1 or for secretion of individual isomers of bTP-1. Significant differences were not detected between the culture conditions even though conceptuses cultured on a rocking platform under 5% CO_2-95% air tend to secrete more

bTP-1 than the two other treatments. Likewise, an increase number of conceptuses is necessary to verify this tendency.

As mentioned above, bTP-1 secretion declined to relatively low levels during the fourth day of culture. If development in culture could be correlated directly with in-vivo development, one would predict that following a 96 h culture period, a Day 18 conceptus would reflect the secretion rate and pattern of bTP-1 complex observed with the Day 22 conceptus. This is not the case. Based on fluorographs from Godkin et al. (1988c), Day 22 bovine conceptuses after the first day of in vitro culture, secrete high levels of bTP-1 in a similar pattern to that detected in the present study . Thus, an improved in-vitro culture system may be required for the maintenance of bTP-1 synthesis and secretion beyond 3 days of culture.

In this study, nine bTP-1 isomers and four additional isomers in the bTP-1 region were identified in conditioned medium from Day 17-18 conceptuses. These 13 isomeric forms were divided in three groups of 4, 4 and 5 isoelectric variants in the 26, 23 and 21 Mr classes, respectively. Nine isomers appeared to be identical to 9 of 10 isomers previously reported to be present in purified bTP-1 and cross-reacting immunologically with anti-oTP-1 (Helmer et al., 1989b). Three of the 4 remaining isomers were detected on fluorographs in studies with radiolabelled bCSP (Geisert et al., 1988a; Godkin et al., 1988c). The most abundant isomers were in the 23 kDa (isomers 5 and 6) and 21 kDa (isomers 9 and 10) Mr range supporting the existence of two dominant molecular weight classes. Previous reports have indicated two major (24 and 22 kDa) and one minor (26 kDa) Mr variants of bTP-1 (Bartol et al., 1985a; Helmer et al., 1987, 1988, 1989b; Anthony et al., 1988; Godkin et al., 1988c; Geisert et al., 1988a). The 22 kDa form was shown to possess a single high-mannose oligosaccharide, whereas the 24 kDa form had a single

complex type oligosaccharide (Helmer et al., 1988). The two dominant Mr forms (23 and 21 kDa), in this study, correspond to the 24 and 22 kDa classes reported earlier. Since the 23 kDa form was always secreted in larger amounts than the 21 kDa form, glycosylation to complex type sugars likely predominates over glycosylation with high-mannose sugars. In addition, at least three isoelectric variants were present in each Mr class, suggesting that each isomeric form is glycosylated differentially. The isomeric forms likely result from different genes since cell-free translation of bTP-1 mRNA produced 4 or 5 isoelectric variants (Anthony et al., 1988).

The bTP-1 complex of proteins are extremely sensitive to proteolytic cleavage (Helmer et al., 1989b). In the current study, bTP-1 purification involved use of Centricon-ultrafiltration units to reduce the time required for processing conditioned medium. Total purification time was as little as 2-3 days, yielding 19.28 μg/culture equivalent of purified bTP-1. Purified bTP-1 contained 9 isomers crossreacting with anti-oTP-1 and a faint 45 kDa contaminant, detected by silver staining. In addition, 1 of 9 bTP-1 isomers was localized in the 19 kDa Mr range. Interestingly, the newly synthesized non-glycosylated form of bTP-1 has a Mr of ~18 kDa (Anthony et al., 1988; Helmer et al., 1988). It should be possible to scale up the purification scheme detailed here by using larger columns and Centricon-ultrafiltration units to purify bTP-1 from larger volumes without affecting overall processing time. The purification scheme of Helmer et al. (1989b) required at least 2 weeks and yielded 20 μg/culture equivalent of highly enriched bTP-1. This enriched bTP-1 complex consisted of 10 isomeric forms crossreacting with anti-oTP-1 and a non-crossreacting contaminant, suspected to be BSA. In this study, the final bTP-1 complex differed from that of Helmer et al. (1989b) in that one bTP-1 isomer (no 10) was missing, a new 19 kDa form was present and the

purity was slightly increased. Lifsey et al. (1988) purified bTP-1 that contained only three immunoreactive isomeric forms in the 21 kDa range.

Bovine TP-1 and oTP-1 are alpha$_{II}$-interferon molecules (Imakawa et al., 1987, 1989; Stewart et al., 1987, 1989b; Charpigny et al., 1988). Godkin et al. (1988b) reported antiviral activity in purified bTP-1, which is characteristic of other interferon molecules, but no details were provided about activity/mg protein. However, purified oTP-1 possesses between 1.3 to 30 x 10^7 U/mg protein (Pontzer et al., 1988; Roberts et al., 1989). In this study, purified bTP-1 contained 1.14 x 10^5 U/mg protein when purified from medium frozen for ~18 mo and 1.49 x 10^7 U/mg protein from medium stored for 6 mo. Storage time may influence bTP-1 biological activity. Additional studies are warranted to determine the best storage conditions for bovine conceptus conditioned culture medium and purified bTP-1.

CHAPTER 4
IMMUNOLOGICAL CROSS-REACTIVITY OF BOVINE TROPHOBLAST PROTEIN-1 AND SYNTHETIC bTP-1 PEPTIDES WITH INTERFERON-LIKE MOLECULES

Introduction

Bovine TP-1, a complex of proteins secreted in large quantity by bovine conceptuses between Days 15 and 25 of pregnancy (Bartol et al., 1985a; Helmer et al., 1987; Geisert et al., 1988a; Godkin et al., 1988c) plays a critical role in recognition of pregnancy in cattle (Knickerbocker et al., 1986a, 1986b; Helmer et al., 1989a, 1989b; Thatcher et al., 1989a). Helmer et al., (1987) defined bTP-1 as a complex of low molecular weight acidic proteins secreted by bovine conceptuses that cross-reacts immunologically with antiserum to ovine trophoblast protein-1 (oTP-1). The bTP-1 complex consists of at least 7 to 9 isomers divided in two predominant molecular weight classes of 22 kDa and 24 kDa (Helmer et al., 1987, 1988, 1989b; Anthony et al., 1988; Godkin et al., 1988b). Bovine TP-1 has been implicated in antiluteolytic mechanisms during early pregnancy because purified bTP-1 decreased in vitro secretion of $PGF_{2\alpha}$ by endometrial explants from Day 17 cyclic cows (Helmer et al., 1989a). Furthermore, cows receiving intrauterine infusions of bTP-1 had extended interestrous intervals and prolonged luteal function when compared to controls (Helmer et al., 1989b). Recent studies have indicated that bTP-1 and oTP-1 are members of the alpha-interferon family of proteins (Imakawa et al., 1987, 1989; Stewart et al., 1987; 1989b; Charpigny et al., 1988). Bovine TP-1

shares 80% amino-acid sequence homology with oTP-1, 50% with BoIFNα_I and 72% with BoIFNα_{II} (Imakawa et al., 1989). In addition, both bTP-1 (Godkin et al.,1988b; Chapter 3) and oTP-1 (Pontzer et al., 1988; Roberts et al., 1989) possess antiviral activity characteristic of interferons.

The present study was to develop immunological probes for studying bTP-1. Because bTP-1 is not available in large quantities, alternatives to conventional immunization schemes for antibody production were explored. In particular, antibodies were developed to three synthetic peptides of bTP-1 and tested for ability to recognize bTP-1 in several immunoassays and for cross-reactivity with other interferon-α's. Other IFN antibodies were tested for cross-reactivity with bTP-1. In addition, peptide fragments of bTP-1 were tested for cross-reactivity with a monoclonal antibody to oTP-1.

Materials and Methods

Materials

Rabbit polyclonal antiserum and mouse monoclonal antibodies to oTP-1 [Ra anti-oTP-1 and Mo anti-oTP-1 (clone HL-100)] were donated by F.W. Bazer. Rabbit polyclonal antiserum against rBoIFN (Ra anti-rBoIFN) was from Genetech (San Francisco, CA) whereas mouse monoclonal and horse polyclonal anti-HuIFN (Mo anti-HuIFN, Ho anti-HuIFN) were from Boehringer Mannheim (Indianapolis, IN). Goat anti-rabbit IgG, sheep anti-mouse IgG and rabbit anti-horse IgG (each as coupled and noncoupled to alkaline phosphatase) were from Sigma Chemical Co. (St. Louis, MO). Monoclonal antibody against 4-azino-2-nitrophenyl hapten (Mo anti-NAP) was from Sera Lab (Crawly Down, Sussex).

Preparation of MEM and supplies for tissue culture were as described in Chapter 3. Bovine CSP and oCSP were obtained as in-vitro secretory products from Day 16-19 bovine conceptuses and Day 15-17 ovine conceptuses (Helmer et al., 1987). Briefly, conceptuses were cultured in either complete MEM or leucine deficient (0.1X) MEM supplemented with 100 μCi [^{3}H]leucine per 15 ml culture medium per 24 h. These secretory proteins were dialysed (6 000-8 000 Mr cutoff) against deionized water containing 0.02% sodium azide or against complete MEM.

Recombinant BoIFN was donated by CIBA-GEIGY (Basle, Switzerland). Purified bTP-1 complex was obtained as described in Chapter 3. Keyhole limpet hemocyanin (KLH), Freund's complete adjuvant and D-PBS were from Sigma Chemical Co. (St. Louis, MO). Protein-A linked to Sepharose-4B was from Pharmacia (Piscathaway, NJ) and 0.22 μm nitrocellulose paper was from Schleicher and Schuell (Keene, NH). XAR-5 X-ray film was obtained from Kodak (Rochester, NY), fast red and napthol phosphate were from Bio-Rad (Richmond, CA) and paraffin was the Paraplast plus brand from Monoject Scientific (St. Louis, MO). Glutaraldehyde was from Polysciences Inc. (Warrington, PA) and the Histocam avidin-biotin immunoperoxidase kit was from Biomeda Corp. (Foster City, CA). Bicinchoninic acid was from Pierce (Rockford, IL).

Synthesis of bTP-1 Fragments

The inferred amino-acid sequence of bTP-1 published by Imakawa et al. (1989) was analyzed for likely surface residues by the surfaceplot computer program produced by Synthetic Peptides Inc. (Richmond Hill, Ontario, Canada). In this program, a combined surface value is based on hydrophilocity, accessibility and flexibility of peptide regions (Janin, 1979;

Karplus and Schulz, 1985; Parker et al., 1986). Three segments of bTP-1 (amino acids 100-113, 131-140 and 152-172) had composite surface values of greater than 50% (Fig. 4-1). These peptides were synthesized by A. Alvarez at the Protein Chemistry Facility of the Interdisciplinary Center for Biotechnology Research at the University of Florida. Synthesis was performed using an Applied Biosystem peptide synthesizer (Model 430A) and solid-phase procedures (Barany and Merrifield, 1980). The amino acid composition of each synthetic peptide was determined using a Beckmann 6300 amino acid analyzer following acid hydrolysis and found to be very similar to the predicted composition. Additionally, each peptide was analyzed by reverse-phase HPLC using a water C_{18} Novapak column and acetonitrile as mobile phase. Each peptide was resolved into one major peak and one or more smaller peaks.

Coupling of bTP-1 Peptides to KLH

Synthetic peptides were dissolved (5 mg/ml) in 100 mM sodium acetate buffer (pH 7.0) containing 5 mg/ml KLH. Glutaraldehyde was added to a final concentration of 7.9 mM and the mixture was gently rocked for 2 h at room temperature before adding sodium borohydrate (16.5 mg as powder) to stop the reaction. After 2 h, the mixture was dialysed (3 500 Mr cutoff) against D-PBS containing 50 U/ml penicillin and 50 µg/ml streptomycin. This mixture was diluted to a final concentration of 1 mg of KLH/ml D-PBS.

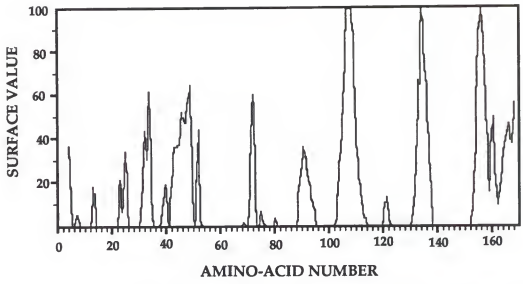


Fig. 4-1. Composite surface values of bovine trophoblast protein-1 based on amino acid sequence published by Imakawa et al. (1989) and as analyzed by the SURFACEPLOT program.

Production of Antipeptide Antibodies

Six females rabbits (2 rabbits/peptide) were immunized with the coupled peptides. Rabbits were bled before immunization to provide preimmune serum (PIS). Five hundred microliters of the peptide-KLH conjugate were prepared as a water-in-oil emulsion with an equal volume of Freund's complete adjuvant. This solution was injected into six subcutaneous and two intramuscular sites at Day 0. Six days later, the rabbits were boosted with 1 ml of a freshly-made solution emulsified in Freund's complete adjuvant. The rabbits were bled via marginal ear vessels at Day 17 and, for two rabbits, at Day 35. The blood samples were allowed to clot for 1 h at room temperature, and at least 2 h at 4°C, before being centrifuged at 2 000 x g for 15 min. The recovered serum was then stored at - 20°C in small aliquots.

Solid-phase ELISA

Fifty microliters of antigen solution (5.5 to 50 µg protein) were pipetted into each well of a polyvinyl flex microtiter plate and incubated for 12 h at 4°C. Plates were washed in PBST [10 mM KPO_4, pH 7.4 containing 0.9% NaCl, 0.05% (v/v) Tween 20 and 0.02% (w/v) NaN_3] and blocked for either 3 h at room temperature or 12 h at 4°C with 200 µl of blocking buffer (10 mg/ml ovalbumin dissolved in PBST). Following two washes, antiserum or negative control serum was added and serially diluted 1:2 or 1:3 across the plates. After a 2-3 h incubation at room temperature and washing three times, species-specific anti-IgG coupled to alkaline phosphatase was added at a 1:1 000 dilution (100 µl) into each well. One hour later, plates were further washed and 100 µl of substrate (1 mg/ml p-nitrophenyl phosphate in 0.1 M glycine-NaOH, pH 10.4 containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$) were added to each well. Absorbance at 405 nm was measured after 60 min with a microtiter plate reader.

Immunoprecipitation

Three samples of [^{3}H]labelled bCSP and [^{3}H]labelled oCSP containing 200 000 dpm were dialysed against water, lyophilized and redissolved in immunoprecipitation buffer [50 mM Tris-acetate pH 7.5, 1 mM PMSF, 1 mM EDTA, 0.3 M NaCl, 2% (v/v) NP-40 and 1 mg/ml BSA]. Fifty microliter of undiluted Ra-anti-oTP-1, Ra-anti-rBoIFN or NRS were added and samples were incubated overnight on a tube turner at 4°C. Subsequently, 100 µl Protein-A Sepharose [10% (v/v) in immunoprecipitation buffer] were added and incubated for 6 h at room temperature on a tube turner. The Sepharose suspension was centrifuged (12 000 x g for 1 min) and washed 4 times in 1 ml washing buffer [50 mM Tris-acetate pH 7.5, 0.3 M NaCl, 0.5% (v/v) NP-40

(v/v), 0.1 % (w/v) SDS and 0.02% (w/v) NaN_3]. Antibody-antigen complexes and the control solution (lyophilized bCSP and oCSP not incubated with antiserum or with NRS) were then dissociated and denatured by boiling for 3 min with 100 µl of loading buffer [62.5 mM Tris-HCl, pH 6.8 containing 5% (w/v) SDS, 10% (w/v) sucrose and 5% (v/v) β-mercaptoethanol]. Tubes with Protein-A Sepharose were centrifuged (12 000 x g; 1 min) and supernatant analyzed by 1-D SDS-PAGE [12.5% (w/v) polyacrylamide] using the buffer system of Laemmli (1970). Radiolabelled proteins were detected by fluorography after soaking gels in 1 M sodium salicylate and drying them.

Western Blotting

Protein solutions were separated according to their molecular weight by 1D SDS-PAGE gels using 12.5% (w/v) polyacrylamide gels and then transferred electrophoretically to nitrocellulose paper using 200 mA current overnight at 4°C in an electroblotting chamber containing 25 mM Tris, 0.2 M glycine and 20% (v/v) methanol. Proteins were loaded onto gels so that they were streaked across the length of the gels (0.3, 1.0 and 1.0 mg protein/16-cm wide gel for rBoIFN, bCSP and oCSP, respectively). Blots were then cut into strips (about 20, 60 and 60 µg protein/strip) and incubated overnight at room temperature in blocking buffer [20 mM Tris-HCl, pH 7.4 containing 1% (w/v) gelatin, 0.5 M NaCl, 0.05% (v/v) Tween 20 and 0.02% (w/v) NaN_3]. Strips of each blot were washed (blocking buffer without gelatin) and then incubated for 2 h with one of the antisera or its control serum diluted 1:100 in incubation buffer [blocking buffer with 0.1% (w/v) ovalbumin instead of 1% gelatin]. Strips were washed for 1-3 h at room temperature and incubated with 1 X 10^6 cpm/ml [^{125}I]labelled-Protein-A (prepared as in Chapter 3). The radiolabelled strips were washed overnight, dried and exposed to Kodak XAR

X-ray film. In an experiment utilizing mouse monoclonal antibodies, two strips were incubated with an anti-mouse IgG antibody coupled to alkaline phosphatase (1:1 000). These two strips were color-developed in a 50:50 (v/v) solution of fast red (6 mg/ml of deionized water) and naphtol phosphate (1 mg/ml in 0.2 M Tris-HCl, pH 9.5 containing 1 mM $MgCl_2$).

Immunocytochemistry

Conceptuses from three pregnant cows at Day 17 of pregnancy and tissues from three cyclic cows (muscle, lung and kidney) and one pregnant cow at Day 17 post estrus (kidney, liver and pancreas) were fixed overnight in Bouin's fixative, dehydrated in ascending concentrations of ethanol and embedded in paraffin. Sections were cut at 5 µm and sequential sections mounted onto gelatin-coated glass slides. Deparaffinized and rehydrated sections were immunostained using avidin-biotin immunoperoxidase kit. Briefly, sections were conditioned for 5-10 min in tissue conditioner provided by the immunoperoxidase kit, blocked with 0.3% hydrogen peroxide for 5 min, and incubated overnight with preimmune serum or antiserum against C-terminal portion of bTP-1 (1:500 dilution in D-PBS containing 1 mg/ml ovalbumin or BSA). On the next day, sections were incubated with a biotinylated goat anti-rabbit IgG for 20-30 min followed by a 20 min incubation with an avidin-horseradish-peroxidase complex. Staining was visualized by 5-10 min incubation with a chromagen solution. The sections were counterstained with hematoxylin. After each step, the sections were washed in PBS (10 mM Na-K-phosphate buffer at pH 7.4, containing 0.15 M NaCl). Antiserum specificity was shown by 6 h preabsorption of the antiserum (1:500 dilution in D-PBS containing 1 mg/ml ovalbumin) with either KLH (1 mg/ml) or 152-172 bTP-1 fragment (100 ng/ml) for 6 h, at room temperature.

Antiviral Assay

The assay is based on inhibition by interferon of viral cytopathic effects (Familletti et al., 1981). Briefly, a monolayer of Madin-Darby bovine kidney (MDBK) cells established in 96-well microtiter plates was incubated for 16-18 h at 37°C with different sample dilutions. The cells were then challenged with bovine vesicular stomatitis virus and incubated for a further 24 h. Cells were fixed and stained with crystal violet to evaluate cell survival. One antiviral unit results in 50% inhibition of cell monolayer lysis.

In one assay, the three peptides alone or mixed together (0.3 mM each in D-PBS) were tested at several dilutions and prepared by making serial threefold dilutions of a starting solution of 0.1 mM peptide. A second assay was performed to test the neutralizing effect of each peptide or mixture of peptides on the antiviral activity of bCSP. For that assay, a bCSP solution was prepared that possessed 20 000 U/ml of antiviral activity. The peptides or the mixture were serially diluted in a constant amount of bCSP (330 U/ml) and added to the wells (0.1 mM starting dilution).

Protein Assay

Protein concentrations were determined by the method of Lowry et al. (1951) or the BCA method by Smith et al. (1985) with BSA as standard.

Results

Cross-reactivity Between Conceptus and other IFNs Using the ELISA

The rabbit polyclonal antibody against oTP-1 reacted against bCSP, oCSP and rBoIFN (Fig. 4-2). The rabbit antiserum against rBoIFN showed a slight positive reaction against bCSP and oCSP when compared to NRS but strongly recognized rBoIFN. Both the mouse monoclonal and equine polyclonal antibodies against HuIFN reacted with rBoIFN. The equine polyclonal antiserum reacted slightly with bCSP and oCSP, whereas the mouse monoclonal antibody did not.

Immunoprecipitation and Western Blotting of oCSP, bCSP and rBoIFN Using Rabbit Polyclonal Anti-oTP-1 and Anti-rBoIFN

Only Ra anti-oTP-1 immunoprecipitated bTP-1 and oTP-1 from conceptus culture medium (Fig. 4-3). A band of radiolabelled protein at Mr 24 kDa (bCSP) or 19 kDa (oCSP) was specifically absorbed with Ra anti-oTP-1. These bands were not present after treatment with NRS or Ra anti-rBoIFN. However, when the more sensitive Western blotting procedure was used (Fig. 4-4), both Ra anti-oTP-1 and Ra anti-rBoIFN recognized the 19 kDa form of oTP-1 as well as a minor lower molecular weight protein. For bCSP, only Ra anti-oTP-1 reacted with bTP-1. Both antisera recognized rBoIFN, reacting with a major form at Mr 18 kDa as well as three other minor molecular weight variants.

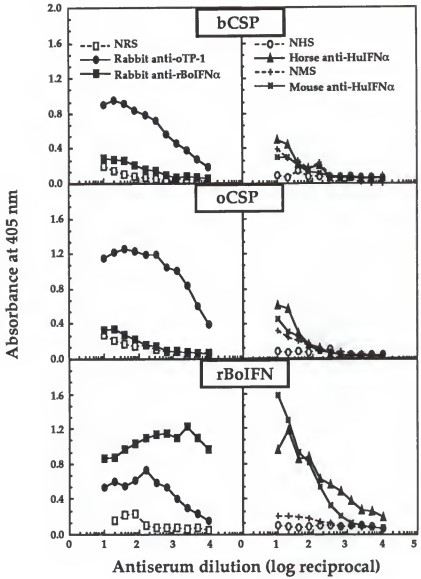


Fig. 4-2. Enzyme-linked immunosorbant assay evaluating immunological cross-reaction of rabbit polyclonal anti-oTP-1, rabbit anti-rBoIFN, horse polyclonal anti-HuIFN and mouse monoclonal anti-HuIFN against 9 μg/well bCSP, 5.5 μg/well oCSP or 0.5 μg/well rBoIFN. Normal rabbit serum (NRS), normal horse serum (NHS) and normal mouse serum (NMS) were used as appropriate negative controls.

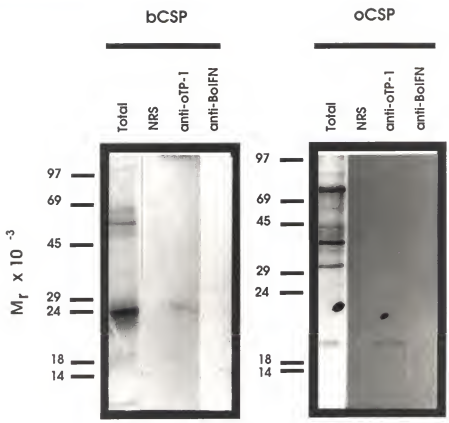


Fig. 4-3. Immunoprecipitation of bovine and ovine conceptus secretory proteins (bCSP and oCSP) with either rabbit anti-oTP-1, rabbit anti-rBoIFN or normal rabbit serum (NRS). The immunoprecipitates and the control solution (total lane; radiolabelled bCSP and oCSP not incubated with serum) were resolved on one-dimensional polyacrylamide gel electrophoresis [12.5 % (w/v) polyacrylamide] and detected by fluorography.

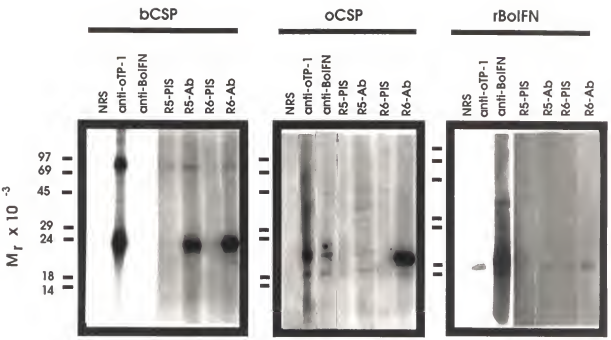


Fig. 4-4. Immunoblotting of bovine and ovine conceptus secretory proteins (bCSP and oCSP) and rBoIFN utilizing rabbit antisera against oTP-1, rBoIFN and the C-terminal portion of bTP-1 [152-172 a.a. fragment; antisera from rabbit 5 and 6 (R5-Ab and R6-Ab)]. Normal rabbit serum (NRS) was used as negative control for anti-oTP-1 and anti-rBoIFN whereas preimmune sera (PIS) of rabbit 5 and 6 were the negative controls for their own antisera. The proteins were separated on one dimensional polyacrylamide gel electrophoresis [12.5% (w/v) polyacrylamide] and blotted against nitrocellulose paper. The blots were cut in small strips (about 20, 60 and 60 µg proteins/strip for rBoIFN, bCSP and oCSP) before being incubated with the respective antiserum.

Screening of Antisera Raised Against the Synthetic Peptides of bTP-1

All the rabbits had an immune response against the carrier protein, KLH (not shown) and the respective peptide used for immunization (Fig. 4-5 to 4-7). Antiserum to peptide 100-113 (Fig. 4-5) reacted slightly or not at all against bCSP and oCSP. The antisera against fragment 131-140 (Fig. 4-6) did not show any cross-reactivity with either bCSP or oCSP. Antisera against the C-terminal peptide (Fig. 4-7) cross-reacted strongly with bCSP and slightly with oCSP (rabbit # 6 only). In another ELISA assay, in which the plates were coated with various amounts of purified bTP-1 (Fig. 4-8), the antiserum against the C-terminal portion of bTP-1 produced a strong dose-dependent colorimetric reaction.

Antisera against fragment 152-172 from rabbits # 5 and # 6 were further screened by Western Blotting (Fig. 4-4, lanes 4-6 for bCSP, oCSP and rBoIFN). As expected, both antisera detected bTP-1 specifically when compared to preimmune serum. Antiserum from rabbit # 6 reacted with a 19 kDa protein (probably oTP-1) in oCSP, whereas antiserum from the other rabbit did not. Neither of the antisera recognized rBoIFN.

Cross-reactivity of a Monoclonal Antibody to oTP-1

In the ELISA assay (Fig. 4-9), monoclonal antibody HL-100, raised against oTP-1, cross-reacted against bCSP, oCSP and very strongly against peptide 152-172. No cross-reactions were detected with either peptides 100-113 or 131-140. Western blotting confirmed that the antibody would react with bTP-1 and oTP-1, but no specific reaction was observed with rBoIFN (Fig. 4-10).

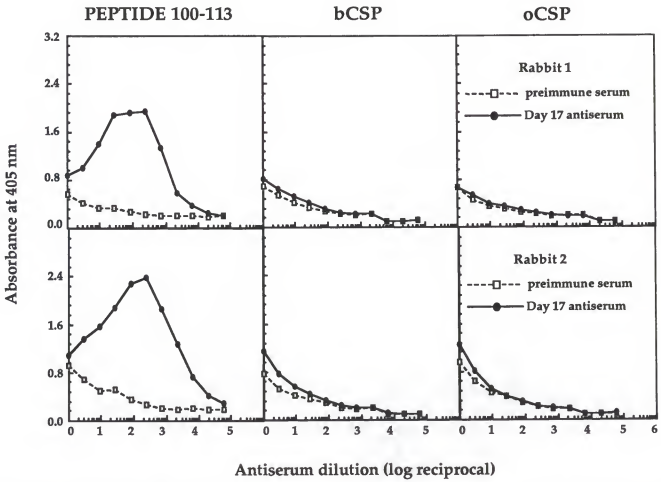


Fig. 4-5. Screening by enzyme-linked immunosorbant assay of two antisera (17 days after immunization) raised against bTP-$1_{100\text{-}113}$ peptide. The ELISA plates were coated with either 50 µg/well 100-113 a.a fragment, 9 µg/well bCSP or 5.5 µg/well oCSP.

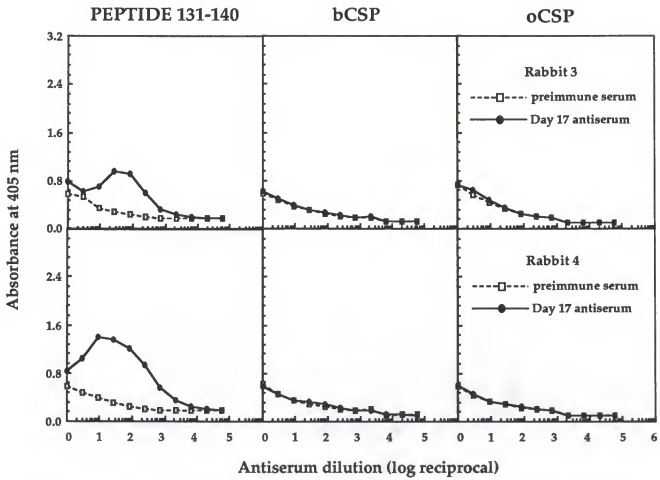


Fig. 4-6. Screening by enzyme-linked immunosorbant assay of two antisera (17 days after immunization) raised against bTP-$1_{131\text{-}140}$ peptide. The ELISA plates were coated with either 50 µg/well 131-140 fragment, 9 µg/well bCSP or 5.5 µg/well oCSP.

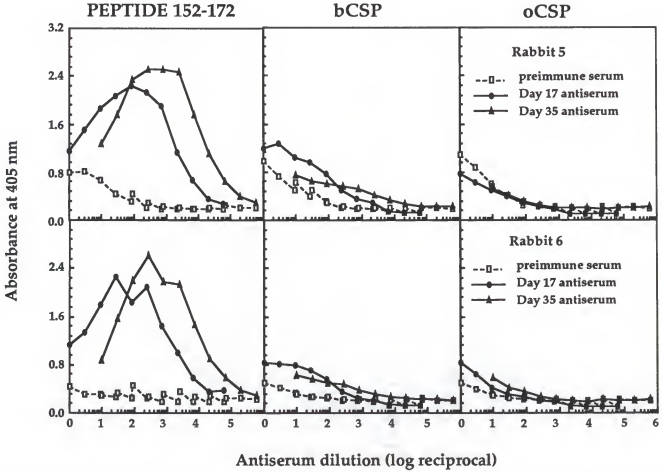


Fig. 4-7. Screening by enzyme-linked immunosorbant assay of two antisera (17 and 35 days after immunization) raised against bTP-$1_{152-172}$ peptide. The ELISA plates were coated with either 50 µg/well 152-172 fragment, 9 µg/well bCSP or 5.5 µg/well oCSP.

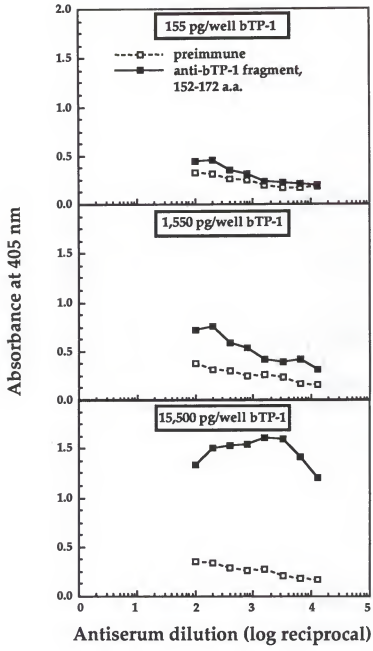
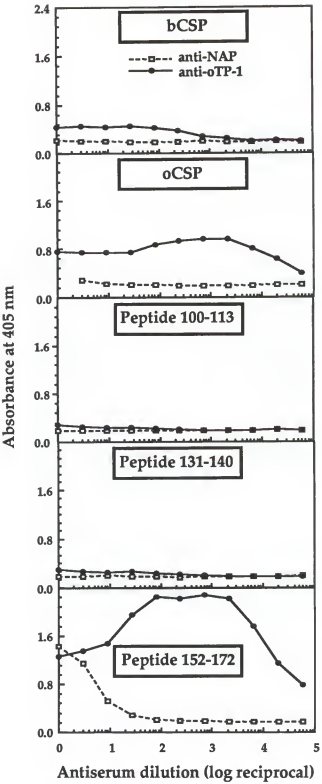


Fig. 4-8. Reaction of antiserum against 152-172 a.a. bTP-1 fragment with purified bTP-1 in enzyme-linked immunosorbant assay. Following coating of the plates with various amount of bTP-1, wells were incubated with a serial dilution of either antiserum or preimmune serum. At the end of the assay, the colorimetric absorbance at 405 nm was proportional to bTP-1 concentration in a dose dependent fashion.

Fig. 4-9. Immunological cross-reactivity between a monoclonal antibody against oTP-1 (clone HL-100; anti-oTP-1) and bCSP, oCSP, and three synthetic bTP-1 peptides using an enzyme-linked immunosorbant assay (ELISA). The monoclonal anti-oTP-1 serum reacted against bCSP and oCSP and very strongly against peptide 152-172 when compared to monoclonal antibody against 4-azino-2-nitrophenyl hapten (negative control; anti-NAP). No cross-reactivity was detected for peptides 100-113 or 131-140.


bCSP
anti-NAP
anti-oTP-1
oCSP
Peptide 100-113
Peptide 131-140
Peptide 152-172
Absorbance at 405 nm
Antiserum dilution (log reciprocal)
2.4
1.6
0.8
0.0
0
1
2
3
4
5

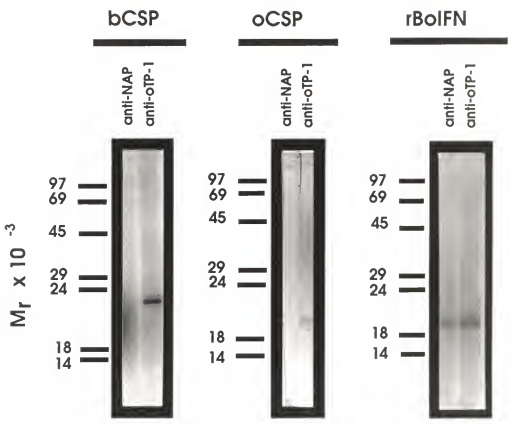


Fig. 4-10. Immunoblotting of bovine and ovine conceptus secretory proteins (bCSP and oCSP) and rBoIFN utilizing monoclonal antibody against oTP-1. Monoclonal antibody against 4-azino-2-nitrophenyl hapten (anti-NAP) was used as negative control for anti-oTP-1. The proteins were separated on one-dimensional polyacrylamide gel electrophoresis [12.5% (w/v) polyacrylamide] and transferred to nitrocellulose before being incubated with the respective antiserum. After incubation with serum the strips were washed and incubated with either radiolabelled protein-A (bCSP and rBoIFN strips) or anti-mouse IgG antibody coupled to alkaline phosphatase (oCSP strips). The radiolabelled strips were washed, dried and exposed to X-ray film. The two other strips were developed in a solution of fast red and naphtol phosphate.

Immunocytochemistry with Antisera Raised Against the 152-172 Peptide Fragment of bTP-1

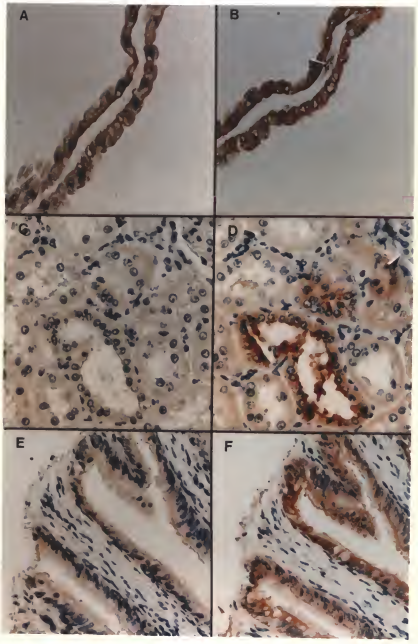
Sections of conceptus tissue from Day 17 was treated with immune serum against peptide 152-172 and immunostaining over all cells of the trophectoderm was detected (Fig. 4-11 B), whereas the extra-embryonic endoderm did stain. A variety of immunocytochemical controls were included to demonstrate specific staining. When preimmune serum was substituted for the immune serum, no staining was detected (Fig. 4-11 A). Staining was also diminished if antiserum against the C-terminal portion of bTP-1 was preabsorbed with the peptide, but was not diminished when the antibody was preabsorbed with KLH (results not shown).

Other tissue sections from Day 17 cyclic (kidney, lung and muscle) or pregnant cows (kidney, liver and pancreas) were also incubated with the immune and preimmune serum. Kidney from either cyclic or pregnant cows showed a positive staining with the antiserum in the renal tubule (Fig. 4-11 C-D). The bronchial epithelium also reacted with the antiserum (Fig. 4-11 E-F), whereas no cells in muscular, hepatic and pancreatic sections stained positively. Staining could not be eliminated with preabsorption of antisera against KLH, but could be decreased by preabsorption with the C-terminal peptide.

Antiviral Activity

Individual peptides or the peptide mixtures had no antiviral activity. Similarly, no single peptide or mixture of peptides could neutralize the antiviral activity of bCSP when 330 U/ml of bCSP were incubated with peptides at concentrations of 15.2 pM to 0.1 mM.

Fig. 4-11. Immunocytochemistry of Day 17 bovine embryos (A-B) and of renal (C-D) and pulmonary tissues (E-F) from a Day 17 cyclic cow using an antiserum against the C-terminal bTP-1 fragment (152-172 a.a.). The sections were immunostained using an avidin-biotin immunoperoxidase kit and counterstained with hematoxylin. Panel A shows a section of a Day 17 bovine embryo incubated with preimmune serum whereas panel B represents a sequential section treated with immune serum. Immunolocalization of bTP-1 was over all cells of the trophectoderm whereas the endoderm was devoid of reaction product (arrow in panel B). Panel C-D represents sequential sections of renal cortex incubated with either preimmune (C) or immune (D) serum. Reaction staining was noticed in the renal tubule but not with the glomerulus (arrow in panel D). Sections of the lung also showed a positive reaction with the immune serum (F) when compared to the section incubated with preimmune serum (E). The bronchiolar epithelium and the cartilage (not shown) were the only two cell types showing a positive staining in the lung.


A
B
C
D
E
F

Discussion

The C-terminal portion (152-172 a.a.) of bTP-1 was the best peptide for production of antisera recognizing bTP-1. This antibody detected bTP-1 in an ELISA, by Western blotting and by immunocytochemistry. The antiserum could probably be used to quantify bTP-1 because it detects bTP-1 in a dose-dependent manner. However, the fact that bTP-1 shares homology with other interferon-like molecules is likely to result in antibodies having relatively low specificity. While there was no evidence that the anti-bTP-$1_{152-172}$ reacted with rBoIFN, immunochemical analyses, detected positive binding of the antibody to antigen(s) in the distal renal tubule and the bronchiolar epithelium, most likely because of the presence of other interferon alpha-molecules related in structure to bTP-1. Localization pattern of bTP-1 in Day 17 bovine trophectoderm agreed with results of previous studies with Day 16 ovine conceptus using a polyclonal antibody against oTP-1 (Godkin et al., 1984a) and with Day 20 bovine conceptus using a polyclonal antibody against bTP-1 (Lifsey et al., 1989).

It was not surprising that antibody to the C-terminal peptide reacted with native bTP-1. Computer analysis indicated a high surface value for these residues and previous studies (Arnheiter et al., 1983) showed that the 10-16 carboxyl-terminal peptide fragments of HuIFNα_I were antigenic and exposed on the surface of the molecule. The monoclonal antibody (HL-100) to oTP-1 also reacted, as expected, with bTP-1. Because the bTP-1 C-terminal peptide reacted with HL-100, it is likely that this antibody is directed to conserved C-terminal epitope. The terminal sequence (152-172) of bTP-1 is 82% homologous to the one for oTP-1 (Imakawa et al., 1989).

Present results confirm that bTP-1 and oTP-1 are interferon-like molecules (Imakawa et al., 1987, 1989; Stewart et al., 1987, 1989b; Charpigny et al., 1988). In the ELISA assays, antisera against oTP-1, rBoIFN and, to a slight extent, HuIFN reacted with bCSP and oCSP. However, while anti-rBoIFN recognized oTP-1 in Western blotting, it did not detect bTP-1. In the immunoprecipitation assay, neither bTP-1 nor oTP-1 were precipitated with anti-rBoIFN. These assay discrepancies could be due to differences in assay sensitivity or in three-dimensional structure of the antigens being altered by assay conditions. The finding that bTP-1 was more reactive to anti-oTP-1 than anti-rBoIFN was expected since oTP-1 shares 80% amino-acid sequence homology with bTP-1 compared to 50% for rBoIFN (Imakawa et al., 1989).

In this study, none of the individual peptides had antiviral or antiviral-neutralizing effects. Ackerman et al. (1984) demonstrated antiviral activity with a HuIFNα_I fragment (111-153), whereas Arnheiter et al., (1981) were unable to show any antiviral activity with three different fragments (71-166, 111-166 and 134-166) of HuIFNα_I. In a recent study, it was shown that three distinct domains (10-35, 78-107 and 123-166) seem to be important for the active configuration of HuIFNα_I and its binding to a cellular receptor (Fish et al., 1989). Additional evidence suggests that no single domain is solely involved in receptor binding and biological activities. Horovitz et al. (1985) demonstrated that two individual HuIFNα_I fragments (1-59 and 112-148), showing no antiviral activity, could inhibit antiviral activity of a HuIFNα_I molecule and that inhibitory activity was enhanced when the two fragments were mixed together.

The difficulty in producing purified bTP-1 (Helmer et al., 1989b) makes the study of its functional properties very arduous. Present results showed that synthetic fragments of bTP-1 may be useful in understanding its

properties. In particular, antiserum against the C-terminal portion of bTP-1 could be employed in different assays to quantify bTP-1 secretion by conceptus tissues. However, care should be applied to the use of this antiserum due to its cross-reactivity with other immunoreactive molecules present in lung and kidney.

CHAPTER 5
INTRAUTERINE AND INTRAMUSCULAR ADMINISTRATION OF RECOMBINANT BOVINE IFN-ALPHA$_{I}$1 PROLONGS LUTEAL LIFESPAN IN CATTLE

Introduction

In sheep and cattle, lifespan of the CL is terminated at the end of the luteal phase of the estrous cycles by endometrial release of $PGF_{2\alpha}$ (Horton and Poyser, 1976). For successful establishment of pregnancy, the conceptus must signal its presence to prevent luteolysis. The critical period for signaling by the conceptus appears to be between 12-13 days after estrus in the ewe (Moor and Rowson, 1966a, 1966b; Hansen et al., 1985) and 15-16 days after estrus in the cow (Betteridge et al., 1980, 1984; Northey and French, 1980; Humblot and Dalla Porta, 1984; Albihn, 1988). In sheep, the molecule produced by the conceptus that acts to prevent luteolysis is oTP-1, which is a polypeptide secreted from days 11-21 of pregnancy (Godkin et al., 1982; Hansen et al., 1985) and which lengthens the interestrous interval in cyclic ewes (Godkin et al., 1984b; Vallet et al., 1988) when infused into the uterus.

In cattle, intrauterine administration of Day 16 (Humblot and Dalla Porta, 1984) or Day 17-18 (Northey and French, 1980) conceptus homogenates to cyclic heifers lengthened the estrous cycle by 3 to 7 days while intrauterine administration of proteins obtained from culture medium conditioned by Day 16-18 conceptuses extended the interestrous interval to 33 days (Knickerbocker et al., 1986b). The molecules responsible for this action are a

complex of proteins called bTP-1 (Bartol et al., 1985a; Helmer et al., 1987; Godkin et al., 1988c). Infusion of bTP-1 into the uterine lumen of cyclic cows extends CL lifespan by inhibiting endometrial $PGF_{2\alpha}$ release in vivo (Helmer et al., 1989a) and inhibiting $PGF_{2\alpha}$ secretion in vitro (Helmer et al., 1989b). In fact, oTP-1 and bTP-1 seem to be biologically active in both species since reciprocal interspecies transfer of bovine and ovine trophoblastic vesicles into uteri of cyclic recipients extends CL lifespan (Martal et al., 1984) and both molecules inhibit in vitro $PGF_{2\alpha}$ release from endometrial explants of Day 17 cyclic cows (Chapter 6, Exp. 4).

Both bTP-1 and oTP-1 have a high amino acid sequence homology with alpha interferons (Imakawa et al., 1987, 1989; Stewart et al., 1987, 1989b; Charpigny et al., 1988). In particular, bTP-1 has a 70% predicted amino acid sequence identity with bovine interferon-α class II (Imakawa et al., 1989). Both bTP-1 (Godkin et al., 1988b; Chapter 3) and oTP-1 (Pontzer et al., 1988; Roberts et al., 1989) possess antiviral activity. In addition, binding of radiolabelled human interferon-α to membrane receptors from uteri of cyclic ewes can be inhibited by purified oTP-1 (Stewart et al., 1987). Furthermore, the secretion of $PGF_{2\alpha}$ and PGE_2 by cultured ovine endometrial cells can be inhibited by both oTP-1 and HuIFN (Salamonsen et al., 1988; 1989). Binding of oTP-1 and rBoIFN to ovine endometrial receptors may differ somewhat, however, since oTP-1 binds to a high-affinity and a low-affinity receptor, whereas rBoIFN binds only to a single high-affinity receptor (Hansen et al., 1989).

Treatment with rBoIFN might delay luteal regression due to its sequence homology with bTP-1. If so, this raises the possibility that interferon treatment may decrease embryonic mortality by supplementing bTP-1 secretion of retarded or small conceptuses with exogenous interferon.

Accordingly, the present experiments were to test whether systemic as well as intrauterine administration of rBoIFN would extend CL lifespan.

Materials and Methods

Materials

Recombinant BoIFN and lyophilized placebo without rBoIFN were kindly provided by CIBA-GEIGY (Basle, Switzerland). Bovine SA (fraction V, protease free) was from Boehringer Mannheim (Indianapolis, IN). Artificial insemination straws and the embryo transfer gun with two subterminal openings on the plastic sheath were purchased from IMV (Minneapolis, MN). The KaMar heat mount detection patches were from KaMar Incorporated (Steamboat Springs, CO), Lutalyse was from the Upjohn Co. (Kalamazoo, MI) and the uterine culture swabs (Accu-CulShure swabs) from Accu-Med Corporation (Pleasantville, NY). The enzyme-linked immunosorbant assay (ELISA) for serum progesterone (Enzynost) was obtained from Hoechst-Russel Agri-Vet (Somerville, NJ). Vacutainer tubes were from Becton Dickinson and Company (Rutherford, NJ).

Exp. 1: Intrauterine Infusions of rBoIFN

Preparation of rBoIFN. Recombinant BoIFN was dissolved in infusion buffer [10 mM $NaPO_4$, pH 7.4 containing 0.9% (w/v) NaCl, 100 IU/ml penicillin, 100 μg/ml streptomycin and 7.5 mg/ml BSA] to a final concentration of 7.5 mg/ml. Solubility of rBoIFN at this concentration was incomplete. Based on protein content of the supernatant fraction using lysozyme as standard (Bradford, 1976), 5.2 mg/ml of the rBoIFN was in

aqueous form. The entire mixture of rBoIFN was filtered through a 0.45 µm sterile filter and prepared for infusion. Antiviral activity of the sterile-filtered rBoIFN solution was estimated to be 10^7-10^8 U/ml (Familletti et al., 1981), where 1 unit of IFN inhibits 50% of the cytopathic effect of vesicular stomatitis virus on Madin-Darby bovine kidney (MDBK) cells.

Infusion buffer was supplemented with additional BSA to give a final concentration of 15 mg/ml and used as a control infusate. These two infusion solutions (BSA and rBoIFN solutions) were loaded into 0.25 ml artificial insemination straws (IMV, Minneapolis, MN). For each straw, 100 µl of the respective solution were aspirated into the middle of the straw. This bolus was lodged between air bubbles. About 50 µl of infusion buffer without BSA were placed at each end of the straw. The straws were plugged and kept at 4°C until used (within less than 8 days).

Intrauterine infusions. A pool of 30 non-lactating dairy cows were palpated rectally and those with a functional CL were injected with 25 mg Lutalyse to regress corpora lutea. Cows were observed for estrus twice daily and the first 10 cows (9 Jersey and 1 Holstein) exhibiting estrus were assigned to the study, housed in an outdoor pen and fed corn silage.

The BSA and rBoIFN solutions were introduced in utero twice daily (0800 and 2000 h) from Days 15.5 (evening) through 21 (morning) after estrus (day of estrus = Day 0). Infusions were with an embryo transfer gun. The cow's rectum was emptied and, while the arm of the inseminator was still inside the rectum, the perineal area was washed thoroughly with antibacterial soap, rinsed and dried with a paper towel. The gun covered by the plastic sheath was placed within a rigid protective sheath before being inserted into the vagina. Epidural anesthesia was administered only when it was difficult to pass the gun through the cervix. The solutions were injected into the

uterine horn ipsilateral to the CL at a location close to the cranial border of the intercornual ligament. Cows were assigned randomly to receive the BSA (n=5) or rBoIFN (n=5) treatment.

Estrus detection and blood sampling. Cows were observed for estrous behavior twice daily (0700 and 1900 h) starting on Day 13 of the estrous cycle. Heat detection was facilitated by the use of KaMar heat mount detection patches. Serum samples were collected from the coccygeal or jugular vein once daily (0730 h) beginning on Day 15 and continuing until detection of estrus for determination of progesterone concentrations.

Bacterial culture and body temperature measurements. Vaginal mucus on the day of estrus was examined carefully for any gross indication of infection. Between Days 0-6 after estrus following intrauterine infusion, cows were evaluated for uterine infection by obtaining uterine cultures with Accu-CulShure swabs. The swabs were brought to the laboratory in less than 1 h, plated and incubated under aerobic and anaerobic conditions.

To determine whether rBoIFN was pyrogenic, rectal temperatures were measured in all cows on one day of the trial at 0, 0.5, 1.0, 3.0 and 5.5 h after uterine infusion of rBoIFN and BSA.

Progesterone determinations. Concentrations of progesterone in serum were measured in duplicate using an ELISA assay and an automatic microtiter spectrophotometer. There was parallelism between inhibition curves for standards and various dilutions of a pregnant cow serum pool. The intraassay and interassay coefficients of variation for four assays were 8.8 and 13.9%, respectively. Limit of detection of the assay (90% of maximal binding) was 0.5 ng/ml.

Statistical analysis. Treatment effects were determined by one-tailed Student's T-test.

Exps 2 and 3: Intrauterine and Intramuscular Administration of rBoIFN

Preparation of rBoIFN. The stock preparation of rBoIFN had a specific antiviral activity of 1.4×10^7 units/mg protein determined by viral plaque inhibition of vesicular stomatitis virus grown on MDBK cells (Horisberger and De Staritzky, 1985).

For intrauterine infusions, a vial containing 125 mg lyophilized rBoIFN was reconstituted in 12.5 ml of a filter-sterilized solution (0.45 μm filter) of 5 mg/ml BSA. The control vial containing the placebo formulation was also reconstituted in 12.5 ml of a sterile solution of 15 mg/ml BSA. Thus, these two solutions contained a final protein concentration of 15 mg/ml (10 mg/ml rBoIFN and 5 mg/ml BSA for rBoIFN infusions; 15 mg/ml BSA for control infusions). Infusion solutions were loaded into 0.25 ml artificial insemination straws. For each straw, 100 μl of the respective solution were aspirated into the middle of the straw. This bolus was bracketed between air bubbles which, in turn, were bracketed between 50 μl of phosphate-buffered saline (10 mM $NaPO_4$, pH 7.4 containing 154 mM NaCl, 100 U/ml penicillin and 100 μg/ml streptomycin). The straws were plugged and kept at 4°C until used (within less than 8 days).

For intramuscular administration, sterile water was added to a vial containing 125 mg rBoIFN to produce a final concentration of 2.5 mg/ml. A solution of BSA was dissolved in the placebo solution to a final concentration of 2.5 mg/ml and was used as control injections.

Intrauterine infusion of rBoIFN (Exp. 2). Fifteen lactating dairy cows maintained in an outdoor lot were palpated rectally and those with functional CL were injected with 25 mg of Lutalyse to regress the CL. Cows were checked twice daily for estrus. The first 10 cows (6 Holstein and 4 Jersey)

exhibiting estrus were assigned randomly to receive control (1.5 mg BSA/infusion; 5 cows) or rBoIFN solutions (1.0 mg rBoIFN with 0.5 mg of BSA/infusion; 5 cows). The control and rBoIFN solutions were introduced in utero, twice daily (0600 h and 1800 h) from Day 14 (morning) through Day 21 (evening) post estrus (day of estrus = Day 0). The infusion technique was as described in the experiment 1 except that no epidural anesthesia was given.

Intramuscular administration of rBoIFN (Exp. 3). Fifty Holstein heifers 11-15 months old, 230 to 343 kg body weight, and kept in four different outdoor pens, were palpated rectally and those with a functional CL injected with 18.5 mg of Lutalyse. Cows were checked three times daily (0700, 1300, 1900 h) for estrous behavior and those detected in estrus (n=23) were assigned randomly within pens to one of four doses of rBoIFN (0, 2.5, 5.0, 10.0 mg/injection). Cows receiving no rBoIFN were given a control injection of 4 ml of BSA (2.5 mg/ml) dissolved in placebo solution. Injections were given intramuscularly twice daily (0700, 1900 h) from Day 14 to Day 21 of the estrous cycle. The experiment was performed on two different occasions, using different heifers to obtain the necessary numbers of cows per group (n = 6, 5, 5, and 7 for 0, 2.5, 5.0 and 10.0 mg, respectively). Therefore, the statistical analysis included effects of period.

Estrus detection and blood sampling. In Exp. 2 and 3, cows were observed twice daily (0700 and 1900 h) for estrus starting on Day 13 of the estrous cycle. Estrus detection was facilitated, on the dairy cows only, by the use of heat mount detectors. Blood samples were collected from the coccygeal vein in a vacutainer tube once daily (between 0600 and 0930 h) beginning on Day 14 of the estrous cycle and continuing until detection of estrus. Blood was allowed to clot at room temperature for 2-3 h and stored an additional 2-6

h at 4°C. The serum was harvested by centrifugation for 10 min at 500 x g and stored at -20°C until assayed for progesterone.

Progesterone determination. Concentrations of progesterone in serum were measured in duplicate, using 100 or 200 µl samples, in a radioimmunoassay described by Knickerbocker et al. (1986b). The intraassay and interassay coefficients of variation for four assays were 7.0 and 9.0%, respectively. Sensitivity of the assay was 31.2 pg/tube.

Statistical analysis. Data analyzed were for interestrous intervals and duration of functional luteal lifespan (the interval between the first day of estrus and the day when serum progesterone concentrations were first below 1 ng/ml). For Exp. 2, data were analyzed by one-tailed Student's t test. For Exp. 3, data were analyzed by the General Linear Models procedure of the Statistical Analysis System (SAS, 1985). The model considered effects of period, pen, treatment, period x pen, period x treatment and pen x treatment. Since very little variation (F value <1) was associated with pen, period x pen, period x treatment and pen x treatment, these effects were subsequently removed from the model. Differences in treatment means were evaluated by orthogonal contrasts (0 vs 2.5, 5.0 and 10.0 mg; 2.5 vs 5.0 and 10.0 mg and 5.0 vs 10.0 mg). Furthermore, cycle lengths and functional CL lifespan were categorized as extended (> 24 days for interestrous intervals and > 23 days for duration of functional luteal lifespan) or normal ($\leq$ 24 days for interestrous intervals and $\leq$ 23 days duration of functional luteal lifespan). Effect of treatment on these distributions was tested by a weighted-least-squares analysis using the CATMOD procedure of the Statistical Analysis System (SAS, 1985). The analysis evaluated treatment effects using orthogonal contrasts as described for least square analysis of variance.

Results

Effects of Intrauterine Infusion of rBoIFN on Interestrous Interval and CL Lifespan

In Exp.1, administration of rBoIFN to cyclic cows from Day 15.5 through 21 extended ($P < 0.02$) the length of the estrous cycle compared to cows receiving BSA (26.8 vs 22.8 days; pooled s.e.m.=1.14 days). Of the five cows receiving rBoIFN, four had cycles of 26 to 30 days (Fig. 5-1). The remaining cow in the rBoIFN-treated group (estrous cycle length=22 days) was most difficult for achieving transcervical passage of the embryo transfer gun and required extensive epidural anesthesia during the trial (4 times). For this particular experiment, functional lifespan of the CL was estimated as the interval between previous estrus and the day when concentrations of progesterone in serum were first less than 1.5 ng/ml (Fig. 5-2). Based on this criterion, rBoIFN extended the functional lifespan of the CL ($P < 0.02$), with intervals being 25.4 days for rBoIFN-treated cows and 21.6 days for the BSA-treated cows (pooled s.e.m.=0.98 days).

In Exp. 2, lactating dairy cows receiving rBoIFN in utero from Days 14 through 21 had estrous cycles of 25 to 36 days in length (Fig. 5-3, panel IU). Four of five cows had cycle lengths greater than 27 days. In contrast, all control cows returned to estrus within 23 to 26 days. Overall, the mean interestrous interval was prolonged ($P < 0.02$) in the rBoIFN-treated group compared to the control group (30.4 vs 24.8 days; pooled s.e.m.=1.41 days).

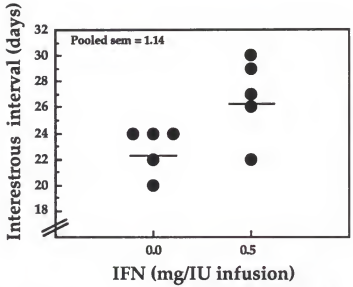


Fig. 5-1. Effect of intrauterine infusion of rBoIFN from Days 15.5 to 21 after estrus on estrous cycle lengths. Circles represent observations for individual cows and the horizontal bar represents means. Cows infused with 0.5 mg/IU infusion of IFN had longer ($P < 0.02$) interestrous intervals than cows that received a control BSA solution.

Recombinant BoIFN also extended ($P < 0.03$) the functional lifespan of the CL (28.4 for rBoIFN-treated cows vs 23.6 for control cows; pooled s.e.m.=1.52 days). Concentrations of progesterone in serum prior to CL regression (i.e., from Day 14-18) were also compared to determine whether rBoIFN affected progesterone secretion before luteolysis; however, values during these five days did not differ between treatment groups. The least-squares means for progesterone for control cows were 7.00, 6.97, 6.89, 7.52 and 7.74 ng/ml at Days 14, 15, 16, 17 and 18, respectively; whereas values for rBoIFN-treated cows were 7.83, 6.71, 8.49, 8.25 and 8.38 ng/ml on the same respective days. The pooled standard error of the mean was 0.58 ng/ml.

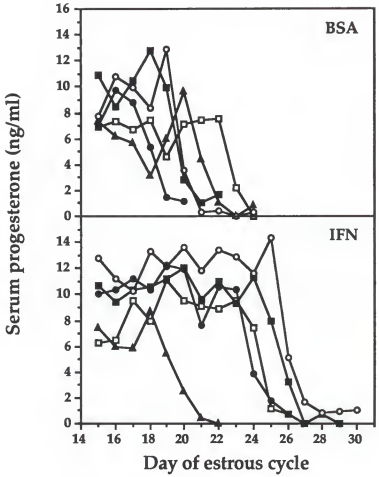


Fig. 5-2. Effect of intrauterine infusion of a control BSA solution (0.0 mg IFN/infusion; Panel A) and rBoIFN (0.5 mg IFN/infusion; Panel B) from Days 15.5 to 21 after estrus on concentrations of progesterone in serum. Plotted are individual profiles from each cow in the experiment. Note that the decline in concentrations of progesterone was delayed in the IFN-treated cows ($P < 0.02$).

Effects of Intrauterine Infusions on Body Temperature and Uterine Infection

In Exp. 1, neither BSA nor rBoIFN administration altered body temperature, which remained between 38.4 and 39.2°C during the 5.5 h examination period after infusion. Based on cultures of uterine swabbings obtained in Exp. 1, none of the cows had evidence of bacterial contamination.

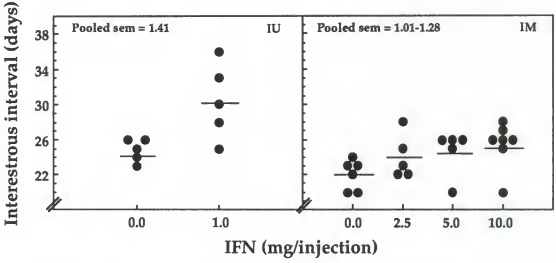


Fig. 5-3. Effect of intrauterine (IU) and intramuscular (IM) administration of various concentrations of rBoIFN on estrous cycle lengths. Circles represent observations for individual cows and the horizontal bar represents means. Cows infused in utero with IFN (Panel IU) had longer ($P < 0.02$) interestrous intervals than cows that received infusion of a control BSA solution. In the IM experiment (Panel IM), the three IFN-treated groups had longer interestrous interval ($P < 0.02$) than the control groups.

Effects of Intramuscular Administration of rBoIFN on Interestrous Interval and CL Lifespan (Exp. 3)

The six heifers receiving 0 mg of rBoIFN had interestrous intervals of 20 to 24 days, whereas those receiving 2.5, 5.0 and 10.0 mg/injection included several heifers with prolonged estrous cycles (2/5, 4/5 and 6/7 heifers with cycle lengths greater than 24 days; Fig. 5-3 , panel IM). The mean interestrous intervals were 22.0 ± 0.9, 24.0 ± 1.0, 24.6 ± 1.0 and 25.4 ± 0.9 days, respectively for heifers receiving 0, 2.5, 5.0 and 10.0 mg of rBoIFN. Interferon-treated

heifers had longer cycles ($P < 0.02$). Cycle lengths did not differ between the 2.5, 5.0 and 10.0 mg/injection groups but tend to increase slightly as dose of rBoIFN increased. Moreover, when results were analyzed as categorical data, orthogonal contrasts confirmed dose-dependent effects of rBoIFN. The number of cows in the rBoIFN-treated groups (2.5, 5.0 and 10.0 mg) with extended cycles was higher ($P < 0.04$) than for the control group. Heifers in the 5.0 and 10.0 mg-treatment groups extended cycles also more frequently than those in the 2.5 mg-treatment group ($P < 0.10$). There was no difference in cycle length for heifers treated with 5.0 and 10.0 mg rBoIFN. Functional lifespan of the CL averaged were 20.5 ± 0.9, 23.0 ± 1.0, 23.6 ± 1.0 and 24.1 ± 0.8 days, respectively for the 0, 2.5, 5.0 and 10.0 mg treated groups. Luteal lifespan was longer ($P < 0.01$) for rBoIFN-treated heifers compared to control heifers, but no different between heifers treated with 2.5, 5.0 and 10.0 mg rBoIFN. When luteal lifespan was analyzed as categorical data, the conclusions were the same as for cycle lengths and with the same probability levels.

Concentrations of progesterone in serum preceding luteolysis (i.e., from Days 14-18) did not differ ($P > 0.10$) between the four treatment groups. The least-squares means of progesterone for heifers receiving no rBoIFN were 8.65, 8.08, 8.36, 9.36 and 7.50 ng/ml on Days 14, 15, 16, 17 and 18, respectively. Interferon-treated heifers, on the same respective days, had values of 9.37, 6.97, 7.86, 9.02 and 8.24 for the 2.5-mg rBoIFN group; 9.34, 8.18, 9.33, 9.88, 7.90 for the 5-mg rBoIFN group; and 9.06, 7.08, 10.13 and 9.75 for the 10-mg rBoIFN group. The pooled standard error of the mean was 0.86 ng/ml.

Discussion

Intrauterine and systemic administration of rBoIFN prolonged functional lifespan of the CL and length of the estrous cycle in cows. Intrauterine infusion of rBoIFN extended interestrous intervals to an average of 26.8 (Exp. 1) to 30.4 (Exp. 2) days, an extension similar to results of intrauterine infusions of Day 16-18 conceptus homogenates (Northey and French, 1980; Humblot and Dalla Porta, 1984) and intrauterine infusions of proteins from conceptus-conditioned culture medium from Days 15.5 through 21 after estrus (Knickerbocker et al., 1986b). Cows receiving intrauterine infusions of BSA had slightly extended interestrous intervals, possibly due to uterine irritation (Yamauchi et al., 1967; Seguin et al., 1974).

Intramuscular administration of rBoIFN also extended interestrous intervals and functional lifespan of the CL by 2 to 4 days. Thus, rBoIFN can be used to cattle to regulate luteal function through an easily accessible route that does not involve cumbersome techniques of intrauterine administration.

In each experiment, a few cows receiving rBoIFN had similar interestrous intervals as control cows; however, the reason for this is unclear. Inadequate dosage is not likely because this phenomenon occurred at all dose levels in Exp. 3 when rBoIFN was administered intramuscularly. Perhaps the mechanisms affecting luteal lifespan extension are more sensitive to rBoIFN in some cows than others.

In cattle, early embryonic mortality decreases the overall pregnancy rate about 30% in normal cows before Days 35-42 (Ayalon, 1978). The period from Days 16 to 21 of gestation may be critical for conceptus survival as the conceptus must be present to prevent luteolysis (Betteridge et al., 1980;

Northey and French, 1980; Humblot and Dalla Porta, 1984) by secreting bTP-1 (Helmer et al., 1989a, 1989b). A Day 16 conceptus, measuring at least 25 mm, can secrete detectable levels of bTP-1 and secretion increases with conceptus development (Geisert et al., 1988a). An optimal amount of bTP-1 may not be secreted when the conceptus is retarded or developing asynchronously with the endometrium. Secretion of bTP-1 is reduced when conceptuses are heat-stressed in vitro (Putney et al., 1988b). Such a response in vivo could decrease conceptus survival through failure to block luteolysis. Thus, clinical application of exogenous bTP-1 or a closely related molecule such as rBoIFN may improve the survival of conceptuses secreting too little bTP-1 during the critical period for maintenance of the CL. The hypothesis, that maintaining CL function pharmacologically during this critical period can be used as a means of increasing fertility, is supported by several lines of evidence. To artificially maintain CL function, Thatcher et al. (1987) administered human chorionic gonadotropin at Day 15 of gestation and improved subsequent pregnancy rates by 1% and 7% on two different farms, respectively. A similar increased in pregnancy rate (11.5%) was obtained following one injection of 10 μg gonadotropin releasing hormone analogue between Days 11-13 post breeding (Macmillan et al., 1986). Heyman et al. (1987) reported a 17% increase in pregnancy rate when a cryopreserved bovine embryo was cotransfered with two trophoblastic vesicles. Therefore, systemic administration of rBoIFN may decrease embryonic loss through its antiluteolytic action.

CHAPTER 6
REGULATION OF PROSTAGLANDIN AND PROTEIN SECRETION FROM ENDOMETRIAL EXPLANTS BY RECOMBINANT BOVINE INTERFERON ALPHA, bTP-1 AND oTP-1

Introduction

In early pregnancy, Day 17-18 bovine conceptuses secrete proteins that prevent luteolysis. The molecules responsible for this action have been identified as a complex of proteins called bTP-1 (Bartol et al., 1985a; Helmer et al., 1987; Geisert et al., 1988a; Godkin et al., 1988c). Infusion of either bCSP (Knickerbocker et al., 1986a, 1986b) or purified bTP-1 (Helmer et al., 1989b) into the uterine lumen of cyclic cows extended CL lifespan by inhibiting endometrial release of $PGF_{2\alpha}$. In-vitro studies support the hypothesis that bTP-1 inhibits release of $PGF_{2\alpha}$. Secretion of $PGF_{2\alpha}$ by endometrial explants collected at Day 17 of the estrous cycle can be inhibited by both bCSP (Gross et al., 1988a) and purified bTP-1 (Helmer et al., 1989a). Explants treated with bTP-1 secreted more PGE_2 than those treated with bCSP (Helmer et al., 1989a), whereas bCSP did not alter PGE_2 secretion (Gross et al., 1988a). The reduction in PGF secretion is associated with induction of an intracellular inhibitor of prostaglandin-synthesizing enzymes called endometrial prostaglandin synthesis inhibitor (EPSI; Gross et al., 1988a; Helmer et al., 1989a). In addition, bCSP, but not bTP-1, induced secretion of several 13-14 kDa proteins by endometrial explants from Day 17 cyclic cows (Gross et al., 1988a; Helmer et al., 1989a).

Bovine TP-1 and oTP-1 share 50% and 70% amino acid sequence homology with bovine interferon α class I and II, respectively (Imakawa et al., 1989). Some interferon alpha molecules possess antiluteolytic activity, but it is unclear whether they act to block luteolysis through mechanisms similar to those induced by bTP-1 and oTP-1. Recombinant BoIFN extended luteal lifespan in cattle when administered into either the uterine lumen or intramuscularly (see Chapter 5). In sheep, continuous intrauterine infusion of 2 mg/day rBoIFN extended the estrous cycle (Lamming et al., 1988; Stewart et al., 1989a). There was no extension of estrous cycle length for cyclic ewes receiving either twice daily intrauterine infusions of 50 μg rHuIFN (Davis and Ott, 1989) or twice daily intramuscular injections of 2 mg rBoIFN (Schalue-Francis et al., 1989). Binding of radiolabelled HuIFN-α to membrane receptors from uteri of cyclic ewes was inhibited by purified oTP-1 (Stewart et al., 1987) and both oTP-1 and HuIFN-α inhibited secretion of $PGF_{2\alpha}$ and PGE_2 by cultured ovine endometrial cells (Salamonsen et al., 1988, 1989). There is, however, evidence for differential binding of oTP-1 and rBoIFN to ovine endometrial receptors with oTP-1 binding to a high-affinity and a low-affinity receptor and rBoIFN binding only to a high-affinity receptor (Hansen et al., 1989).

The present study utilized in-vitro approaches to determine whether rBoIFN acts through a similar mechanism as bTP-1 to cause CL maintenance. Endometrial explants from Day 17 cyclic cows, cultured in presence of rBoIFN, were analyzed for secretion of trichloroacetic acid-precipitable radiolabeled macromolecules, secretion of PGF and PGE_2, induction of endometrial prostaglandin synthesis inhibitor (EPSI) and enhanced endometrial secretion of proteins with Mr of 13-14 kDa.

Materials and Methods

Materials

DL-[4, 5-^{3}H]leucine (SA=26 Ci/mmol), L-[^{35}S]methionine (SA=1 300-1 400 Ci/mmol), [5, 6, 8, 11, 12, 14, 15-^{3}H]$PGF_{2\alpha}$ (SA=160-180 Ci/mmole) and [5, 6, 8, 12, 14, 15-^{3}H]PGE_2 (SA=~140-170 Ci/mmole), ^{125}Iodine (SA=~169 mCi/μg) were from Amersham Corp. (Arlington Heigths, IL). Preparation of MEM and supplies for tissue culture and electrophoresis were as described in Chapter 3. Arachidonic acid and human serum albumin (fraction V; HSA) were from Sigma Chemical Co. (St.Louis, MO) and BSA was from either Boehringer Mannheim, Indianapolis, IN (fraction V, protease free; Exp. 1, 2 and 3) or Sigma Chemical Co., St. Louis, MO (fractionV; Exp. 4). Recombinant BoIFN and lyophilyzed rBoIFN placebo were kindly provided by CIBA-GEIGY (Basle, Switzerland). Recombinant HuIFN from Hoffman-LaRoche (Nutlen, NJ; Roferon; HuIFN-α_I2) was dialysed (3 500 Mr cutoff) against complete MEM before use. Preparation of Day 17-18 bCSP for endometrial culture was as described by Gross et al. (1988a). Purified bTP-1 dissolved in D-PBS was produced as described in Chapter 3. Ovine TP-1 was purified as described by Godkin et al. (1982) and dialysed against 10 mM Tris, pH 8.2, containing 0.05 M NaCl. Dialysis tubing (3 500 and 6 000-8 000 Mr cutoff) was purchased from Spectrum Medical (Los Angeles, CA). Antiserum to PGF was a gift from T.G. Kennedy and antiserum to PGE_2 was provided by E.L. Lilly Co. (Indianapolis, IN).

Endometrial Culture

Experiment 1. Four cycling Brangus cows were slaughtered at Day 17 post-estrus (Day 0 = day of estrus) and their reproductive tracts quickly removed within 30 min and flushed with 40 ml MEM. All cows had a functional CL based on its size and texture. Caruncular and intercaruncular endometria from the uterine horn ipsilateral to the CL were dissected from myometrium and cut into small pieces, mixing the two types of endometrial tissue (1-3 mm^3). For prostaglandin and EPSI analyses, endometrial tissues were cultured in quadruplicate in 100 mm petri dishes containing 500 mg tissue in MEM supplemented with 0.2 mg arachidonic acid per 15 ml MEM and either 5 μg/ml BSA or 5 μg/ml rBoIFN. For each treatment, two dishes were incubated for 24 h and two dishes for 48 h before harvesting medium and tissue. For the 48 h incubation, medium was changed and replaced after 24 h with the same amount of fresh MEM (15 ml) and treatment proteins. Medium (500 μl) was collected from each dish at 3, 6, 12, 18 and 24 h and, when applicable, also at 30, 36, 42, and 48 h of incubation. An additional set of two dishes was prepared for protein analyses. Procedures were as described above except that 250 mg endometrium was cultured in 60 mm petri dishes for 24 h. The MEM (7.5 ml/culture) was leucine deficient (0.1X) and contained 0.1 mg arachidonic acid and 50 μCi [^{3}H]leucine per dish. Aliquots (200 μl) of medium were collected at 3, 6, 12, 18 and 24 h of incubation. All incubations were performed on a rocking platform at 38.5°C for 24 h or 48 h under an atmosphere of 50:47.5:2.5 (v/v/v) nitrogen:oxygen:carbon dioxide. At the conclusion of incubation (24 or 48 h), tissue and medium were separated by centrifugation (700 x g, 10 min) and stored at -20°C until analyzed.

Experiment 2. Four Brangus cows were killed on Day 17 of the estrous cycle and endometrium was harvested as previously described except that the uterus was not flushed with MEM. For prostaglandin analysis, duplicate tissue samples (500 mg) were cultured in 15 ml MEM containing 0.2 mg arachidonic acid and 25 μg/ml BSA, 25 μg/ml BSA containing rBoIFN placebo, 25 μg/ml rBoIFN or 10% bCSP (13.5 ml MEM and 1.5 ml bCSP medium). An additional duplicate set of tissues (250 mg) for protein analyses was cultured in 7.5 ml medium deficient in methionine (0.1X) and containing 0.1 mg arachidonic acid and 25 μCi [^{35}S]methionine. Tissues were cultured for 24 h under the usual conditions. At the end of the incubation period, tissue and medium were separated by centrifugation (1 464 x g, 15 min, 4°C). The 500 mg-medium samples were stored at -20°C until analyzed whereas the 250 mg-medium samples were loaded into dialysis tubing for determination of incorporation of methionine into polypeptides.

Experiment 3. Three cyclic Brangus cows were slaughtered on Day 17 after estrus and endometrium was obtained as described in Exp. 2. Duplicate tissue samples (500 mg) were placed into 100 mm petri dishes containing 0.2 mg arachidonic acid in 15 ml MEM prepared to provide one of the following treatments: 1) medium alone without additional protein; 2) BSA at 4.8, 24 or 120 μg/ml; or 3) rBoIFN at 4.8, 24 or 120 μg/ml. Tissues were cultured for 24 h under the same conditions as for Exps 1 and 2. Samples of medium (500 μl) were taken at 6, 12, 18 and 24 h of incubation. At the end of the culture period, tissue and medium were separated by centrifugation (1 464 x g, 30 min, 4°C) and stored at -20°C until analyzed.

Experiment 4. Three cyclic Senepol cows and 1 cyclic Holstein cow were slaughtered on Day 17 after estrus. All cows had a functional CL weighing 2.7 to 4.6 g wet weight and serum concentrations of progesterone

from 3.0 to 6.9 ng/ml. Endometrial explants were obtained as described in Exp. 2. Unlike previous experiments, endometrial explants were collected from uterine horn ipsilateral and contralateral to the CL and washed 3 times in fresh MEM before use. Duplicate 250 mg tissue cultures were performed in 7.5 ml for each of the nine treatments (Table 6-1). Explants were incubated at 38.5°C for 24 h under an atmosphere of 50:45:5 (v/v/v) nitrogen:oxygen:carbon dioxide. Samples of medium (500 µl) were taken at 0, 12 and 24 h of incubation. At the end of the culture period, tissue and medium were separated by centrifugation (1 464 x g, 15 min, 4°C) and stored at -20°C until analyzed for prostaglandins.

Table 6-1. Description of the nine treatments for endometrial explant culture in Exp. 4. The MEM for every treatment contained rBoIFN placebo (1:13 000 dilution of 10 mg/ml placebo formulation), 0.02 mM Tris-HCl (pH 8.2), 0.1 mM NaCl, 1/61 (v/v) D-PBS, 0.1 mg arachidonic acid and various amounts of proteins as shown in the table. The additives were prepared so that all cultures contained identical amounts of salt, BSA and HSA except for medium alone where no extra albumin was added. BSA=bovine serum albumin, HSA=human serum albumin, rBoIFN=recombinant bovine interferon alpha$_I$, oTP-1=ovine trophoblast protein-1, bTP-1=bovine trophoblast protein-1, bCSP=bovine conceptus secretory proteins, rHuIFN=recombinant human interferon alpha$_I$.

Treatment	BSA µg/ml	HSA µg/ml	rBoIFN µg/ml	oTP-1 µg/ml	bTP-1 µg/ml	bCSP µg/ml	rHuIFN µg/ml
1. Medium alone	-	-	-	-	-	-	-
2. Control	50	15	-	-	-	-	-
3. 10% bCSP	50	15	-	-	-	38.9	-
4. 42.0 nM bTP-1	50	15	-	-	1.00	-	-
5. 4.20 nM bTP-1	50	15	-	-	0.10	-	-
6. 0.42 nM bTP-1	50	15	-	-	0.01	-	-
7. 42.0 nM oTP-1	50	15	-	0.80	-	-	-
8. 42.0 nM rBoIFN	50	15	0.76	-	-	-	-
9. 42.0 nM rHuIFN	50	15	-	-	-	-	0.76

Secretion Rate of Radiolabelled Proteins

Incorporation of radiolabelled leucine into de novo secreted proteins in Exp. 1 was determined by trichloroacetic acid (TCA) precipitation (Mans and Novelli, 1961). Duplicate 50 µl aliquots of medium were dried onto Whatman 3 MM paper previously saturated with 20% (w/v) TCA. Proteins were precipitated onto the filter paper by serial washings with 20% (w/v) TCA, 5% (w/v) TCA and 95% ethanol. Radioactivity of precipitated proteins was determined by scintillation spectrometry. For Exp. 2, secretion of [^{35}S]methionine-labelled proteins was estimated by quantifying the amount of radiolabel incorporated into nondialysable macromolecules. Conditioned medium was extensively dialysed (3 changes of 4 l; Mr cutoff=6 000-8 000) against deionized water containing 0.02% (w/v) NaN_3, 1 mM PMSF and 1 mM EDTA. Radioactivity of non-dialysable [^{35}S]labelled proteins was determined directly by scintillation spectrometry.

Secretion Rate of 13-14 kDa Proteins (Exp. 2)

Aliquots (100 000 cpm) of dialysed [^{35}S]labelled conditioned medium were lyophilyzed and separated using 2D SDS-PAGE as described in Chapter 3. Briefly, the samples were run in the first dimension by isoelectric focusing in 4.2% (w/v) polyacrylamide tube gels containing 2.5% (v/v) pH 3-10, 1.8% (v/v) pH 5-7 and 0.8% (v/v) pH 9-11 ampholytes. Tubes gels were subjected to electrophoresis in the second dimension using 12.5% polyacrylamide slab gels in the presence of SDS and β-mercaptoethanol. Gels were stained with Coomassie Blue and prepared for fluorography by soaking in 1 M sodium salicylate, drying and exposing to Kodak XAR film at -70°C. All films were developed for the same period of time. They were photographed and five protein spots in the 13-14 kDa region were punched from the gel, using the

fluorograph as the template. Spots were placed directly into scintillation vials and acrylamide gel dissolved in 500 µl 30% hydrogen peroxide at 70°C for 48 h. After removing vials from the oven and cooling them, 100 µl 500 mM ascorbic acid was added and the vials stored in the dark for 48 h before being counted for radioactivity. Radioactivity in each spot was corrected mathematically to a per culture basis.

Assay for Inhibition of Prostaglandin Synthesis

Cotyledonary microsomes from parturient cows, containing prostaglandin synthesizing enzymes, were prepared as described elsewhere (Gross et al., 1988b). Within-cow, replicates of endometrial tissues (Exps 1 and 3) were pooled and homogenized with a Dounce homogenizer in ice-cold 0.05 M Tris-HCl (pH 7.4) containing 0.25 M sucrose (1 g tissue/1 ml). The homogenates were centrifuged at 13 600 x g for 15 min at 4°C and supernatants were then ultracentrifuged at 100 000 x g for 60 min at 4°C to yield a high-speed cytosolic supernatant. The generating system (0.5 ml; 500 mg tissue equivalent of cotyledonary microsomes) was incubated with 100 µg arachidonic acid (100 µl) and 500 µl of either 0.1 M potassium phosphate buffer (pH 7.5) or endometrial cytosolic supernatant (500 mg tissue equivalent). Final incubation volume was brought to 2 ml with 0.1 M potassium phosphate buffer (pH 7.5). All incubations were for 1 h at 37 to 39°C on a rocking platform and terminated by adding 250 µl 95% ethanol. Samples were centrifuged (1 500 x g, 20 min, 4°C) and supernatants frozen at -20°C until assayed for PGF.

Radioimmunoassay (RIA) Procedures

The PGF assay was described and validated by Gross et al. (1989b). Unextracted samples from either the inhibitor assay (2.5 and 200 µl) or endometrial culture medium (50 and 100 µl) were assayed in duplicate. Antiserum against $PGF_{2\alpha}$ had 94% crossreactivity with $PGF_{1\alpha}$; 2.4% with PGE_2 and less than 0.1% with 13,14-dihydro-15-keto-$PGF_{2\alpha}$, PGE_1 and arachidonic acid. Due to high cross-reactivity with $PGF_{1\alpha}$, results of the assay are presented as PGF. Interaassay and intraassay coefficients of variation were 12.2% and 18.5%, respectively.

The PGE_2 assay also was described and validated by Gross et al. (1988b). Unextracted samples of endometrial culture medium (50 µl) were assayed in duplicate. Antiserum against PGE_2 crossreacted 24% with PGE_1, 1.7% with $PGF_{2\alpha}$ and less then 0.1% with 13,14-dihydro-15-keto-$PGF_{2\alpha}$, $PGF_{1\alpha}$, 6-keto-$PGF_{1\alpha}$ and arachidonic acid. Interaassay and intraassay coefficients of variation were 10.3% and 21.6%, respectively.

Statistical Analysis

Data were analyzed statistically via least squares analysis of variance using the General Linear Model of the Statistical Analysis System (SAS, 1985). Data from endometrial culture experiments on incorporation of radiolabelled materials into secreted proteins (Exp. 1) and prostaglandin secretion (Exps 1-3 and 4) were analyzed considering effects of cow, treatment, cow x treatment, replicate (cow x treatment), time, cow x time, treatment x time, cow x treatment x time and residual. Differences in treatments were evaluated by orthogonal contrasts. In Exp. 2, data on secretion of proteins in the 13-14 kDa Mr range, and secretion rates for PGF and PGE_2 were analyzed using the following model: cow, treatment, cow x treatment and residual. Data for

inhibition of PGF in the prostaglandin generating system by cytosol of endometrial explants (Exps 1-3) were analyzed considering effects of cow, treatment, cow x treatment and residual.

Results

Incorporation of Radiolabel into Proteins

In Exp. 1, the TCA-precipitable [^{3}H]labelled proteins from endometrial explants of Day 17 cyclic cows (Fig. 6-1, panel A) increased during time of culture ($P < 0.001$). While overall secretion rates for radiolabelled proteins was similar between rBoIFN and BSA cultures, there was a treatment by time interaction ($P < 0.07$) due to lower accumulation of protein for rBoIFN-treated explants during the second 12 h of culture. In Exp. 2 (Fig. 6-2, panel A), endometrium cultured with rBoIFN secreted less nondialysable [^{35}S]labelled proteins ($P < 0.08$) than BSA-rBoIFN placebo cultures whereas 10% bCSP had no effect when compared to BSA controls.

Secretion Rate of 13-14 kDa Proteins

In Exp. 2, five isoelectric variants of a 13-14 kDa protein, with pIs of ~9.0, 8.2, 7.0, 6.6, and 6.0, were identified following 2D SDS-PAGE and fluorography (Fig. 6-3) and analyzed quantitatively for rates of secretion (Fig. 6-2, panel B). Ten percent bCSP did not alter secretion rate for any of the 5 isoelectric variants when compared to BSA cultures, but rBoIFN decreased ($P < 0.05$) secretion rates for two proteins (proteins 4 and 5, pI ~ 6.6 and 6.0) when compared to BSA+rBoIFN placebo.

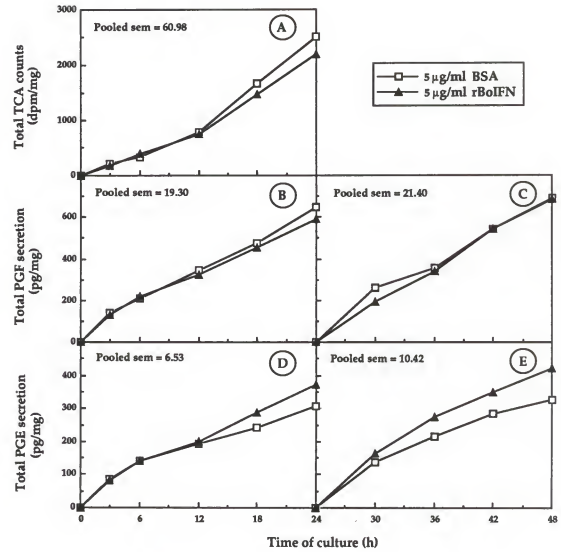


Fig. 6-1. Daily secretion of TCA-precipitable [^{3}H]labelled proteins and prostaglandins by endometrial explants from Day 17 cyclic cows (n=4) treated with either 5 µg/ml BSA or 5 µg/ml rBoIFN (Exp. 1). Results are the mean accumulated secretion during the first (n=16; panel A, B, D) and second days of culture (n=8; panel C, E). The secretion rate of TCA-precipitable proteins and prostaglandins increased with time of culture ($P < 0.001$). The overall secretion rate of labelled proteins was similar between rBoIFN and BSA cultures, but there was a treatment x time interaction ($P < 0.07$). Rates of endometrial PGF secretion were similar during the first and the second 24 h of culture with no interaction between treatments and time of culture. Endometrial explants cultured with rBoIFN secreted more PGE_2 during the second 24 h of culture ($P < 0.03$) and there was a treatment x time interaction for the first ($P < 0.001$) and second 24 h ($P < 0.05$) of culture.

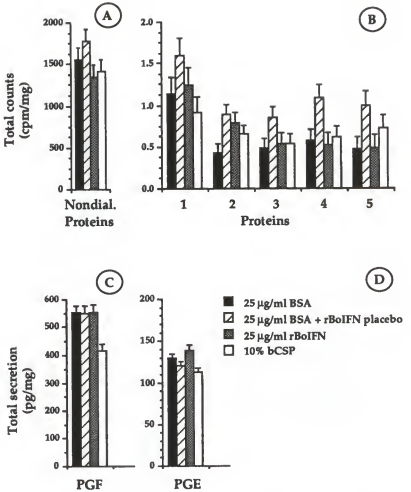


Fig. 6-2. Daily endometrial secretion of nondialysable [^{35}S]labelled proteins (panel A), five proteins falling into the 13-14 kDa range (panel B; pI of ~ 9.0, 8.2, 7.0, 6.6, and 6.0 for protein 1 to 5, respectively), and prostaglandins (panel C and D; Exp. 2). Endometrial explants from Day 17 cyclic cows (n=4) were incubated in duplicate for 24 h in medium containing 25 µg/ml BSA, 25 µg/ml BSA+rBoIFN placebo, 25 µg/ml rBoIFN or 10% bCSP. Columns represent least-squares means and the vertical bar, the s.e.m. Endometrium cultured with rBoIFN secreted less nondialysable radioactive proteins ($P < 0.08$) than BSA-rBoIFN placebo cultures whereas 10% bCSP had no effect when compared to BSA controls. Recombinant BoIFN decreased secretion rates ($P < 0.05$) for two proteins (proteins 4 and 5) when compared to appropriate controls, whereas bCSP did not alter the secretion rate of any of the five proteins. Bovine CSP reduced PGF secretion ($P < 0.01$) compared to the BSA control, whereas rBoIFN had no effect as compared to BSA+rBoIFN placebo control. Based on appropriate orthogonal contrasts with the controls, rBoIFN increased PGE_2 secretion ($P < 0.05$) and bCSP lowered PGE_2 secretion ($P < 0.05$).

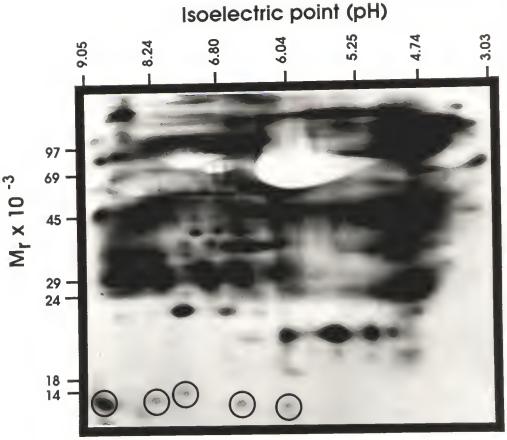


Fig. 6-3. Representative fluorograph of two-dimensional gel electrophoretogram of dialysed [^{35}S]labelled-conditioned medium from an endometrial explant culture of a Day 17 cyclic cow incubated with 25 µg/ml rBoIFN. An aliquot (100 000 cpm) of dialysed medium was lyophilyzed and run in the first dimension by isoelectric focusing and in the second dimension by molecular weight using 12.5% (w/v) polyacrylamide gel. Secretion rates of five isoelectric variants of a 13-14 kDa protein, identified from left to right as protein 1 to 5, were analyzed quantitatively (see Fig. 6-2, panel B).

Prostaglandin Synthesis by Endometrial Explants

In Exp. 1, endometrial accumulation of PGF (Fig. 6-1, panel B-C) increased with time ($P < 0.001$), but both treatments resulted in similar secretion rates during the first and second 24 h of culture. In addition, there was no time of culture by treatment interaction. Endometrial explants cultured with rBoIFN secreted more PGE_2 (Fig. 6-1, panel D-E) during the second 24 h of culture ($P < 0.03$) than BSA-treated explants. There was also a treatment x time interaction for the first ($P < 0.001$) and second 24 h of culture ($P < 0.05$) because rate of accumulation of PGE over time was greater for rBoIFN-treated than control explants.

In Exp. 2, PGF in medium after 24 h of culture averaged 552, 550, 551 and 414 pg/mg tissue for cultures treated with BSA, BSA+rBoIFN placebo, rBoIFN and 10% bCSP (pooled s.e.m.=25), respectively. Bovine CSP reduced PGF secretion ($P < 0.01$) compared to the BSA control, whereas rBoIFN had no effect compared to BSA+rBoIFN placebo. Average accumulation of PGE_2 in medium after 24 h of culture was 129, 120, 139 and 112 pg/mg (pooled s.e.m.=5) for BSA, BSA+rBoIFN placebo, rBoIFN and 10% bCSP, respectively. Based on appropriate orthogonal contrasts with the controls, rBoIFN increased PGE_2 secretion ($P < 0.05$) and bCSP lowered PGE_2 secretion ($P < 0.05$).

In Exp. 3, PGF accumulation was similar for all treatments (Fig. 6-4, panel A) and increased in a linear fashion during the time of culture ($P < 0.001$) but without a treatment by time interaction. Endometrium treated with rBoIFN secreted more PGE_2 ($P < 0.001$; Fig 6-4. panel B) than endometrium treated with medium alone or with BSA and the rate of accumulation of PGE_2 over time was greater for rBoIFN cultures (treatment x time interaction, $P < 0.03$). Endometrium treated with 4.8 μg/ml rBoIFN secreted less PGE_2 ($P < 0.10$) than that treated with 24 and 120 μg/ml rBoIFN.

There was no effect of BSA on PGE_2 secretion by endometrium when compared to that in medium alone or medium with BSA. Concurrently with the present experiment, endometrial explants were cultured in presence of 10% bCSP and results were reported by Helmer et al. (1989a). Briefly, bCSP inhibited PGF, but did not alter PGE_2 secretion when compared to the controls. These published results indicate that the endometrial explant cultures were sensitive to agents that inhibit prostaglandin production.

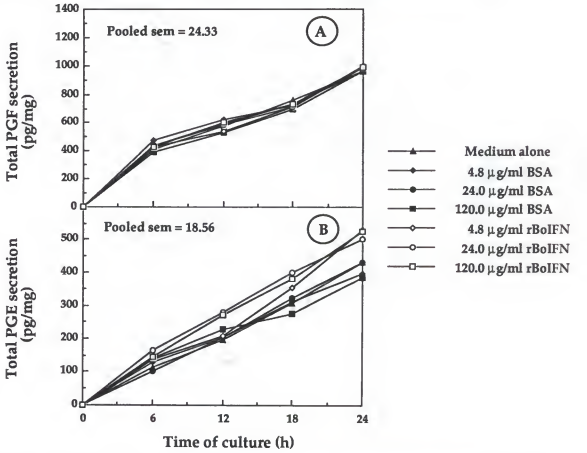


Fig. 6-4. Prostaglandin secretion by endometrial explants from Day 17 cyclic cows (n=3) incubated for 24 h in medium containing no BSA, 4.8 to 120 µg/ml BSA or 4.8 to 120 µg/ml rBoIFN (Exp. 3). The secretion rate of prostaglandins increased with time of culture ($P < 0.001$). Secretion of PGF was not affected by treatment, but PGE_2 secretion was higher for rBoIFN treated cultures (treatment, $P < 0.001$ and treatment x time, $P < 0.03$) compared to medium alone or BSA. In addition, secretion of PGE_2 was lower with 4.8 µg/ml rBoIFN than with 24 and 120 µg/ml rBoIFN ($P < 0.10$). There was no effect of BSA on PGE_2 secretion when compared to medium alone or medium with BSA.

Results from Exp. 4 (Fig. 6-5) differed from Exps 1-3 in several ways. First, unlike the other experiments, endometrial explants secreted higher levels of PGE_2 than PGF. Second, PGF and PGE_2 secretion from cultures with medium alone was less ($P < 0.002$) than for all other treatments, all of which included BSA. All interferon-like molecules (bCSP, bTP-1, oTP-1, rBoIFN and rHuIFN) inhibited PGF and PGE_2 secretion compared to control cultures ($P < 0.001$), which contained the same amounts of BSA, HSA and salts. For secretion of PGF, there was a treatment x time interaction ($P < 0.01$) because differences between control and treated cultures varied over time. Overall PGF secretion for each interferon-treated cultures did not differ from each other. The bCSP- and bTP-1-treated explants secreted more PGE_2 ($P < 0.07$) than the oTP-1-, rBoIFN- and rHuIFN-treated explants. Additional comparisons between treatments did not detect differences in amounts of PGE_2 secreted or treatment x time interaction.

Intracellular Inhibitor of PGF Synthesis in Endometrial Explants

In two experiments (Exps 1 and 3; Fig. 6-6), the generating system produced 5.40 (s.e.m. of 0.045; panel A) and 3.80 (s.e.m. of 0.082) ng PGF/h/500 mg tissue equivalent of cotyledonary microsomes. Cytosolic supernatants from endometrium treated with BSA and rBoIFN inhibited the generating system in Exps 1 ($P < 0.01$; panel A) and 3 ($P < 0.01$; panel B) equally. Assays for inhibition of PGF secretion in the prostaglandin generating system were considered valid since cytosolic supernatant from bCSP-treated endometrial explants was tested at the same time and shown to decrease PGF synthesis. In Exp. 1, PGF synthesis was reduced 17 and 20% when cytosol preparations were from endometrial explants cultured with 10% bCSP for 24 and 48 h,

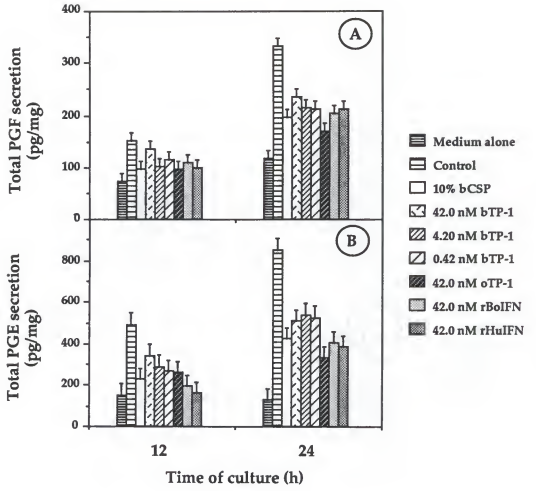


Fig. 6-5. Prostaglandin secretion by washed endometrial explants from Day 17 cyclic cows (n=4) incubated for 24 h in medium containing various levels of different IFN-like molecules (Exp. 4). Columns represent least-squares means and the vertical bar, the s.e.m. Endometrium in medium alone secreted less PGF ($P < 0.001$) and PGE_2 ($P < 0.002$) than that in other treatments. The bCSP, bTP-1, oTP-1, rBoIFN and rHuIFN inhibited PGF and PGE_2 release compared to the control treatment ($P < 0.001$). For secretion rates of PGF, there was a treatment x time interaction ($P < 0.01$), but overall PGF secretion by endometrium among IFN-like cultures did not differ. The bCSP- and bTP-1-treated endometrial explants secreted more PGE_2 ($P < 0.07$) than the oTP-1-, rBoIFN- and rHuIFN-treated endometrial explants.

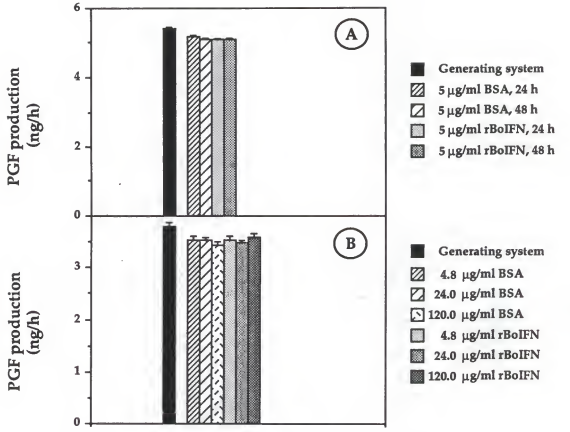


Fig. 6-6. PGF synthesis by a cotyledonary microsomal preparation incubated with either buffer or an aliquot of a high speed endometrial cytosolic supernatant. Panel A (Exp. 1) and B (Exp. 3) represent experiments in which endometrial explants were incubated with different treatments and collected after 24 (Exps 1 and 3) or 48 h (Exp. 1). The endometrial cytosol from BSA-treated explants in Panels A and B (500 mg tissue equivalent/assay) inhibited PGF synthesis by the microsomal preparation ($P < 0.01$), but no differences were detected between BSA and rBoIFN.

respectively (Gross et al., 1988a). In Exp. 3, cytosol from explants treated with 12.7 μg bCSP/ml decreased PGF synthesis by 42% (Helmer et al., 1989a).

Discussion

Results of the present study do not clearly elucidate effects of rBoIFN on PGF and PGE_2 by endometrium. Results from Exps 1-3, in which endometrium was treated similarly to that described by Gross et al. (1988a) and Helmer et al. (1989a), indicated that rBoIFN had no effect on PGF, but increased PGE_2 secretion compared to medium alone or medium containing BSA. Absolute concentrations of prostaglandins were similar to that previously reported data; higher secretion rates for PGF than PGE_2 and no effect of BSA on prostaglandin secretion. Results from EPSI analysis also support previous results in magnitude of PGF synthesis. However, unlike bCSP and bTP-1, rBoIFN did not induce the synthesis of EPSI (Gross et al., 1988a; Helmer et al., 1989a), even though these experiments were done under identical conditions (i.e., in same assay) as reported for EPSI induction by bCSP and bTP-1 (Gross et al., 1988a; Helmer et al., 1989a).

Results from Exp. 4, are different from the other experiments. Compared to endometrial explants with no additives, prostaglandin secretion was enhanced in cultures with BSA, HSA and various salts. The total amount of PGF secretion was less in Exp. 4 than Exps 1-3 and more PGE_2 was secreted relative to PGF. The relative changes in secretion from 12 to 24 h of culture were small. In this experiment, all treatments with interferons decreased PGF and PGE_2 secretion. Perhaps albumin is necessary for rBoIFN to inhibit prostaglandin secretion by protecting rBoIFN from proteolysis or

providing necessary cofactors. Washing of endometrial explants in Exp. 4 may have removed unknown factors which otherwise block the activity of rBoIFN. Last, results of Exp. 4 could have been flawed due to some unknown factor; however, results from Exp. 4 do agree with results of Salamonsen et al. (1988, 1989) that oTP-1 and rHuIFN decreased PGF and PGE_2 secretions from ovine endometrial cell cultures.

Infusion of purified bTP-1 into the uterine lumen of cyclic cows extends CL lifespan and inhibits endometrial release of $PGF_{2\alpha}$ (Helmer et al., 1989b). In-vitro studies suggest that bTP-1 blocks endometrial synthesis of $PGF_{2\alpha}$. Purified bTP-1 decreased in-vitro secretion of PGF by endometrial explants from Day 17 cyclic cows (Helmer et al., 1989a). Recombinant BoIFN also extended luteal lifespan in cattle when administered in utero (see Chapter 5). Results of the fourth experiment suggested that, as for bTP-1, rBoIFN could block the endometrial secretion of $PGF_{2\alpha}$. However, results of the first three experiments indicated that rBoIFN increases endometrial secretion of PGE_2 without altering PGF secretion. Purified bTP-1 also increased endometrial secretion of PGE_2 in-vitro (Helmer et al., 1989a). In cattle, Gimenez and Henricks (1983) extended CL lifespan with intrauterine infusion of PGE_2 from Days 9-12 of the estrous cycle. In another study, intrauterine infusion of PGE_2 and estradiol from Days 13 to 21 also prolonged luteal lifespan compared to cows receiving vehicle, estradiol or PGE_2 (Reynolds et al., 1983). Prostaglandin E_2 may act synergistically with estradiol to directly or indirectly extend CL lifespan. In-vitro studies have indicated that PGE_2 has a luteotrophic effect on bovine luteal cells by increasing the synthesis of progesterone (Speroff and Ramwell, 1970; Marsh, 1970). In mid-cycle, large bovine luteal cells, which constitute approximately 40% of the luteal tissue, secrete most of the circulating progesterone (O'Shea et al., 1989).

These large luteal cells are very responsive to PGE_2 (Fitz et al., 1984a, 1984b). Prostaglandin E_2 is also a potent vasodilator and can increase utero-ovarian blood flow (Thatcher et al., 1984b). Around Day 15 of pregnancy, an increase in uterine blood flow coincides with an increase in circulating progesterone which inhibits the luteolytic effect of endometrial $PGF_{2\alpha}$ (Reynolds, 1986).

Synthesis of [^{3}H] and [^{35}S]labelled proteins by endometrial explant cultures was decreased by rBoIFN compared to control BSA- ([^{3}H]labelled cultures) or BSA+rBoIFN placebo- ([^{35}S]labelled cultures) treated endometrial explants. Similar results were obtained with bCSP and bTP-1 (Gross et al., 1988a; Helmer et al., 1989a). Recombinant BoIFN decreased endometrial secretion of two of the 13-14 kDa Mr proteins, whereas bCSP had no effect on secretion of these proteins. Previous results indicate that secretion of these 13-14 kDa proteins was enhanced by bCSP (Gross et al., 1988a; Helmer et al., 1989a), but not by bTP-1 (Helmer et al., 1989a), and elevated at Day 17 of pregnancy (Geisert et al., 1988a; Gross et al., 1988a). However, Bartol et al. (1985b) did not detect effects of pregnancy on these proteins. Quantifying amounts of secreted radiolabelled proteins by gel punching and scintillation spectrometry is less subjective than evaluating fluorographs with the naked eye and may provide better estimates of labelled protein concentration than intensity readings with a densitometric scanner. In addition, low Mr proteins identified here and elsewhere, which vary slightly in pI and Mr from one study to another, could be different molecules induced inconsistently under various experimental conditions. It is also possible that incorporation of [^{35}S]methionine and [^{3}H]leucine into these proteins is different and gives different secretion patterns.

In-vivo studies indicated that intramuscular and intrauterine administration of rBoIFN prolonged interestrous intervals. In the present in-

vitro study, it is unclear whether rBoIFN inhibits endometrial synthesis of PGF through the same mechanism as bTP-1.

CHAPTER 7
IN-VIVO EFFECTS OF INTRAUTERINE AND INTRAMUSCULAR ADMINISTRATION OF rBoIFN ON ESTROUS CYCLE LENGTH, OVARIAN FUNCTION AND OXYTOCIN-INDUCED PGFM RELEASE IN COWS

Introduction

Bovine TP-1 and oTP-1 are proteins secreted in large quantity by Days 15-22 bovine conceptuses (Bartol et al., 1985a; Helmer et al., 1987; Geisert et al., 1988a; Godkin et al., 1988c) and by Day 13-21 ovine conceptuses (Godkin et al., 1982), respectively. These proteins are critical signals for prevention of luteolysis and act by depressing endometrial $PGF_{2\alpha}$ secretion (Godkin et al., 1984b; Vallet et al., 1988; Helmer et al., 1989a, 1989b). They are members of the alpha-interferon family (Imakawa et al., 1987, 1989; Stewart et al., 1987, 1989b; Charpigny et al., 1988) and possess antiviral and immunosuppressive activity characteristic of other IFN molecules (Godkin et al., 1988b; Pontzer et al., 1988; Newton et al., 1989b; Niwano et al., 1989; Roberts et al., 1989; Chapter 3).

It is likely that non-conceptus IFNs can simulate the antiluteolytic effect of bTP-1 and oTP-1 when given to cyclic animals. Administration of rBoIFN in utero (0.5-1.0 mg/infusion) or intramuscularly (2.5-10.0 mg/injection) on Day 14-21 to cycling cows prolonged interestrous intervals and CL lifespan (Chapter 5). In sheep, continuous intrauterine infusion of 2 mg/day rBoIFN from Day 9 to 19 extended luteal lifespan and decreased circulating levels of PGFM (Stewart et al., 1989a). Data from in-vitro studies support the antiluteolytic action of non-conceptus. Human IFN inhibited

secretion of $PGF_{2\alpha}$ by ovine endometrial cells (Salamonsen et al., 1988, 1989) as did rBoIFN in one of four experiments with cultures of bovine endometrial explants (Chapter 6). Non-conceptus IFNs can suppress prostaglandin synthesis in other tissues. Synthesis of PGE_2 was reduced when human mononuclear leucocytes (Dore-Duffy et al., 1983) or human peripheral monocytes (Browning and Ribolini, 1987) were preincubated with alpha-interferons. In addition, alpha-interferons decreased prostaglandin formation by mouse macrophages by diminishing liberation of arachidonic acid from phospholipids (Boraschi et al., 1985).

Other reports indicate that alpha-interferon may attenuate circulating levels of steroid hormones. In women, administration of IFN reduced serum estradiol and progesterone concentrations (Kauppila et al., 1982) while in men, serum testosterone concentrations diminished 1 to 11 h after a subcutaneous injection of IFN (Orava et al., 1986; Orava, 1989). In addition, pretreatment with IFN reduce hCG-stimulated testosterone secretion from cultured porcine Leydig cells (Orava et al., 1985). Intramuscular injection of rBoIFN can also induced hyperthermia in cattle (Bielefeldt Ohmann and Babiuk, 1986; Newton et al., 1989a) and this hyperthermia could affect endocrine and reproductive functions (Biggers et al., 1987; Putney et al., 1988a, 1988b, 1989a, 1989b).

Regression of CL in cyclic cows involves a close hormonal interaction between the ovary and uterine endometrium. Experimental data indicates that follicular estrogens stimulate events leading to pulsatile release of an uterine luteolysin, $PGF_{2\alpha}$. Prostaglandin $F_{2\alpha}$ is the accepted luteolytic agent in cattle and its secretion from the endometrium is monitored by measuring peripheral concentrations of 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM) (Kindahl et al., 1976, 1981; Guilbault et al., 1984; Knickerbocker et al., 1986c).

Administration of estradiol during the mid-luteal phase stimulates PGF secretion as detected by an increase of $PGF_{2\alpha}$ in uterine fluids (Bartol et al., 1981b), higher concentrations of $PGF_{2\alpha}$ and PGFM in the utero-ovarian vessels (Knickerbocker et al., 1986c) and an increased concentration of circulating PGFM (Bartol et al., 1981b; Thatcher et al., 1986b). Uterine blood flow was also increased following estradiol administration (Knickerbocker et al., 1986c). Furthermore destruction of ovarian follicles around Days 9-15 of the cycle extends luteal lifespan (Fogwell et al., 1985; Hughes et al., 1987) and prevents complete luteolysis of CL after injection of exogenous $PGF_{2\alpha}$ (Hughes et al., 1987). McCracken et al. (1981, 1984b) hypothesized, using sheep as the experimental model, that ovarian estradiol initiates luteolysis by increasing synthesis of endometrial oxytocin receptors. Subsequently, oxytocin, presumably secreted by the CL and posterior pituitary in cattle (Wathes et al., 1983), binds to these receptors to stimulate pulsatile release of $PGF_{2\alpha}$. During early pregnancy, endometrial release of $PGF_{2\alpha}$ is attenuated in response to estrogen (Rico et al., 1981; Thatcher et al., 1984b) and oxytocin (Lafrance and Goff, 1985; Putney et al., 1989b). Diminished PGF responses were also observed following estradiol injection on Day 18 of the cycle, of cows receiving intrauterine infusions of bCSP from Day 15.5 to Day 21 (Knickerbocker et al., 1986a).

In the present study, we tested the hypothesis that intramuscular and intrauterine administration of rBoIFN extends luteal lifespan by reducing uterine release of $PGF_{2\alpha}$. Release of PGFM in response to oxytocin was analyzed to determine whether rBoIFN inhibits endometrial prostaglandin synthesis. Additionally, effects of rBoIFN on circulating progesterone and estradiol concentrations at Days 14-17, body temperature at Days 14-17, and

follicular characteristics at Day 17 were ascertained to evaluate potential side effects.

Materials and Methods

Materials

Recombinant BoIFN and placebo for rBoIFN were provided graciously by CIBA-GEIGY (Basle, Switzerland). Bovine serum albumin (fraction V), heparin and penicillin-streptomycin were from Sigma Chemical Co. (St Louis, MO). Dulbecco's phosphate buffered saline was from Gibco (Grand Island, NY). Lutalyse was from Upjohn Co. (Kalamazoo, MI), and oxytocin from Vedco Company (Arcadia, CA). Artificial insemination straws of 0.5 ml-volume were purchased from IMV (Minneapolis, MN). The artificial insemination gun with one terminal opening on the plastic sheath, as well as the second protective plastic sheaths, were from Nasco (Fort Atkinson, WI). Vacutainer tubes were from Becton Dickinson (Rutherford, NJ), intravenous catheters (14 gauge; 5.5 inches long) from Abbott Hospitals (North Chicago, IL) and intravenous extension sets from Cole-Parmer (Chicago, IL). Elasticon was obtained from Johnson and Johnson (New Brunswick, NJ). Linear scanning ultrasound (LS-300 Ultrasound Diagnostic System) with a 7.5 MHz probe was from Tokyo Reiki Co., Ltd (Tokyo, Japan).

Preparation of rBoIFN

Solutions were prepared as described in Exp. 2 and 3 of Chapter 5. Briefly, intrauterine solutions contained either: a) 10 mg/ml rBoIFN supplemented with 5 mg/ml BSA or b) 15 mg/ml BSA dissolved in rBoIFN

placebo. These two solutions were loaded into 0.5 ml artificial insemination straws. For each straw, 200 µl of the respective solution was aspirated into the middle of the straw. The bolus was bracketed between air bubbles which, in turn, were bracketed between 100 µl of D-PBS containing 100 IU/ml penicillin and 100 µg/ml streptomycin. Intramuscular injection solutions were comprised of 2.5 mg/ml rBoIFN or 2.5 mg/ml BSA dissolved in the placebo solution.

Administration of rBoIFN

Twenty five non-lactating dairy cows were palpated per rectum, and those with a functional CL were injected with 25 mg Lutalyse. Cows were checked three times daily for behavioral estrus. Fifteen cows (12 Holstein and 3 Jersey) that exhibited estrus were assigned randomly to receive one of three treatments (5 cows/treatment) from Days 14 to 17 of the estrous cycle. Control cows received 3 mg BSA/intrauterine infusion and 10 mg BSA/intramuscular injection. In the second group, cows received both intrauterine infusions of 2 mg rBoIFN plus 1 mg BSA and an intramuscular injection of 10 mg BSA. The third group received an intrauterine infusion of 3 mg BSA and an intramuscular injection of 10 mg rBoIFN. The BSA and rBoIFN solutions were administered twice daily (0600 and 1800 h). Intrauterine infusions were performed as described in Chapter 5 with approximately one-half of the solution deposited in the right horn and the other half in the left horn.

Estrus Detection and Blood Collection

Cows were observed for estrus three times daily (0700, 1200 and 1900 h) beginning on Day 14 of the estrous cycle. Blood samples (20 ml) were collected from coccygeal vessels into heparinized vacutainer tubes, twice daily

(0530 and 1200 h) from Day 14 to 17, and once daily (morning) from Day 18 to estrus. In addition, at 0900 h on Day 17, cows were fitted with a jugular catheter connected to an intravenous extension set. The catheterization system was secured to the animals neck through Elasticon raps. The catheter was flushed with heparinized saline (200 U/ml) after each sampling to prevent occlusion. Frequent blood sampling (20 ml) was performed every 15 min for 1 h before (1000 to 1100 h) and 2 h after (1100 to 1300 h) an intravenous challenge with 100 U oxytocin at 1100 h. An additional sample was obtained 5 min after oxytocin (1105 h). All samples were collected in heparinized tubes (25 U), put on ice and the plasma was harvested within 1 h by centrifugation at 12 000 x g for 15 min. Plasma was aspirated and stored at -20°C until analyzed.

Radioimmunoassay

Plasma samples were assayed for progesterone as described in Exps 2 and 3 of Chapter 5. The intraassay and interassay coefficients of variation (CV) for three assays were 8.7 and 3.8%, respectively. Plasma collected from Day 14 to estrus also was analyzed for concentrations of estradiol by a previously validated radioimmunoassay (Guilbault et al., 1987). The intraassay and interassay CV were 28.2 and 15.6%, respectively. Plasma samples obtained on Day 17 (1000 to 1300 h) were analyzed for PGFM as described by Guilbault et al. (1984). The intraassay and interassay CV were 7.9 and 16.8%, respectively.

Ovarian Ultrasound

Real time linear scanning ultrasonic examination was performed at 0800 h on Day 17. Before examination, fecal material was removed from the

rectum. The transducer was then inserted rectally and moved laterally over the ovaries to examine ovarian sequential sections for follicular populations. Size and frequency of occurrence of follicles greater than 3 mm were recorded.

Body Temperature Measurements

Rectal temperatures were measured on all cows at 0550, 0900, 1200 and 1730 h on Day 14 and at 0550 and 1200 h from Days 15 to 17.

Statistical Analysis

Data were analyzed by least squares analysis of variance using the General Linear Models procedure of the Statistical Analysis System (SAS, 1985). Interestrous intervals, duration of functional luteal lifespan (interval between the first day of estrus and the day when serum progesterone concentration first fell below 1 ng/ml) and duration of proestrus (interval between the day when concentrations of serum progesterone first fell below 1 ng/ml and the day of behavioral estrus) were analyzed with treatment as the only effect in the model. Orthogonal contrasts were used to compare rBoIFN-treated cows to control cows and to compare rBoIFN-IU to rBoIFN-IM. Effects of treatments on concentrations of estradiol and progesterone, and rectal temperature were analyzed considering effects of treatment, cow(treatment), day, day x treatment, day x cow(treatment), time, treatment x time, time x cow(treatment), day x time and treatment x day x time. Data for rectal temperature on Day 14 and PGFM concentrations on Day 17 (1000 to 1100 h pre-oxytocin samples, and 1100 to 1300 h post-oxytocin samples) were analyzed using a split-plot analyses of variance with repeated measurements over time and a model with effects of treatment, cow(treatment), time, treatment x time and time x cow(treatment). Because of heterogeneity of

variance among treatments, PGFM data were transformed using square roots prior to analysis. Tests of homogeneity of regression were performed to describe whether rBoIFN altered time trends in concentrations of PGFM from 1100 to 1300 h. As an additional test of whether rBoIFN altered response to oxytocin, an analysis was performed to determine whether the within-animal variance in time trends (determined in large part by the response to oxytocin) differed between treatments. For each treatment group, untransformed data for PGFM were analyzed by least-squares analysis of variance using model components of cow, time and cow x time. The F tests for heterogeneity of cow x time for each treatment comparison (i.e., control vs IM, control vs IU, IM vs IU) were calculated as the ratio of mean-square errors of cow x time.

Data on the size of the largest follicle were analyzed considering treatment as the only main effect. Follicles identified by ultrasound were categorized as class 1, 2 and 3 follicles by having diameters of 3-5 mm, 6-9 mm and > 9 mm, respectively. Number of follicles were analyzed by either least squares analysis of variance using effects of treatment, cow(treatment), class and treatment x class, or by the CATMOD procedure (SAS, 1985) considering effects of treatment, class and treatment x class.

Results

Effects of rBoIFN on Interestrous Interval, Corpus luteum Lifespan and Proestrous Interval

Intrauterine (IU) or intramuscular (IM) administration of rBoIFN to cyclic cows from Days 14 through 17 extended length of the estrous cycle ($P < 0.001$; Fig 7-1) and CL lifespan ($P < 0.02$; 21.8, 24.0 and 25.4 days for control,

rBoIFN-IU and rBoIFN-IM, respectively; pooled s.e.m.=0.77) compared to cows receiving BSA. Cows treated with rBoIFN intramuscularly had a longer interestrous interval ($P < 0.03$) than cows treated with rBoIFN in utero. However, CL lifespan did not differ due to route of rBoIFN administration. There were no differences in length of proestrus among the three treatment groups (0.8, 1.0 and 1.6 days for control, rBoIFN-IU and rBoIFN-IM, respectively; pooled s.e.m.=0.45).

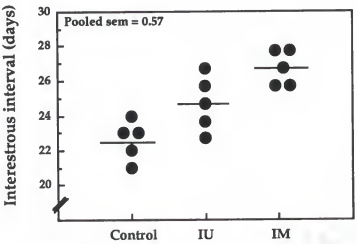


Fig. 7-1. Effect of intramuscular (IM) and intrauterine (IU) administration of rBoIFN from Days 14 to 17 after estrus on estrous cycle lengths. Cows assigned randomly to one of three treatments (5 cows/treatment) received a control BSA solution, 2 mg rBoIFN in utero (IU) or 10 mg rBoIFN intramuscularly (IM). Cows were challenged on Day 17 with 100 U oxytocin. Circles represent observation for individual cows and the horizontal bar represents means. Cows receiving rBoIFN IU or IM had extended ($P < 0.001$) interestrous intervals compared to cows receiving a control solution. Cows injected intramuscularly with rBoIFN had longer interestrous intervals ($P < 0.03$) than cows infused in utero with rBoIFN.

Effects of rBoIFN on Body Temperature

Body temperature from Days 14 to 17 (Fig. 7-2, panel A) was affected by treatment ($P < 0.005$), day ($P < 0.001$), treatment x time ($P < 0.03$), day x time ($P < 0.001$) and treatment x time x day ($P < 0.005$). These effects resulted because

both types of rBoIFN caused an acute increase in body temperature on Day 14, with peak values at 6 h after administration. Intramuscular rBoIFN administration was more pyrogenic than intrauterine infusion ($P < 0.01$). On other days examined, rBoIFN had no effect on body temperature.

Effects of rBoIFN on Plasma Progesterone

Overall means for concentrations of progesterone from Days 14 to 17 (Fig. 7-2, panel B) did not differ between the three treatment groups. Nonetheless, there were treatment x time ($P < 0.005$), day x time ($P < 0.03$) and treatment x time x day interactions ($P < 0.005$), similar to that for body temperature. At Day 14, rBoIFN-IM caused an acute decrease in circulating progesterone coinciding with the increase in body temperature. On Day 15, progesterone again decreased following administration of rBoIFN-IM, but this decrease was not as pronounced as on Day 14. Progesterone levels also decreased slightly on Days 16 and 17 following rBoIFN-IM. Administration of rBoIFN-IU did not affect concentrations of progesterone in plasma.

Effects of rBoIFN on Ovarian Characteristics

Overall, there was no evidence that rBoIFN affected follicular characteristics. Means for size of the largest follicle on Day 17 were 10.2, 11.6 and 11.6 mm (pooled s.e.m.=1.23) for control, rBoIFN-IU- and rBoIFN-IM-treated cows, respectively. Average numbers of follicles per cow classified in class 1, 2 and 3 were 7.00, 0.80 and 0.60 for control cows; 4.20, 1.80 and 1.00 for rBoIFN-IU treated cows; and 5.20, 1.00 and 1.40 for cows receiving rBoIFN-IM (pooled s.e.m.=0.78). Treatments and treatment x class effects were not significant.

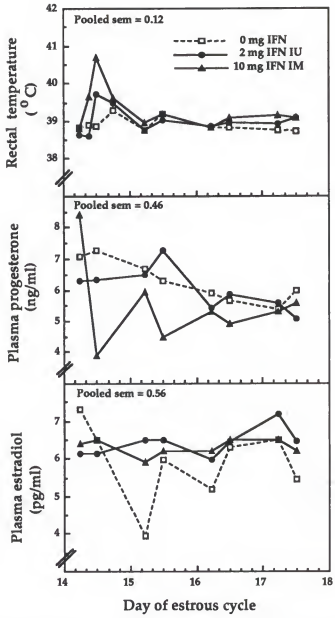


Fig. 7-2. Effect of intrauterine (IU) and intramuscular (IM) administration of rBoIFN on rectal temperature and concentrations of progesterone and estradiol in plasma. Body temperatures from Days 14 to 17 (panel A) were affected by treatment ($P < 0.005$), day ($P < 0.001$), treatment x time ($P < 0.03$), day x time ($P < 0.001$) and treatment x time x day ($P < 0.005$). Analysis of data from Day 14 only demonstrated that rBoIFN induced hyperthermia in cyclic cows ($P < 0.01$) compared to control cows. Intramuscular administration of rBoIFN was more pyrogenic than intrauterine administration ($P < 0.01$). Concentrations of progesterone (panel B) were affected by time ($P < 0.005$), day x time ($P < 0.03$) and treatment x time x day ($P < 0.005$). Concentrations of estradiol (panel C) were not affected by treatment.

In spite of low means level of estradiol at Day 15 for cows receiving BSA (Fig. 7-2, panel C), concentrations of estradiol were not significantly affected by any main effects or interactions.

Effects of rBoIFN on Oxytocin-Induced Release of PGFM

Individual cow profiles of concentrations of PGFM in plasma are shown in Fig. 7-3 and average concentrations of PGFM in Fig. 7-4. Based on analysis of variance and heterogeneity of regression analysis on transformed data, concentrations of PGFM before oxytocin were similar among groups, but after oxytocin, PGFM concentrations increased ($P < 0.001$). For 4 of 5 control cows, 2 of 5 rBoIFN-IU and 1 of 5 rBoIFN-IM treated cows PGFM concentrations increased after the oxytocin challenge (Fig. 7-3). Analysis of PGFM values, from 0 to 120 min post injection by regression analysis, indicated that the response for rBoIFN-treated cows was lower than for control cows (Fig. 7-4; $P < 0.05$). Additionally, rBoIFN-treated cows had a decreased variation over time in their response to oxytocin ($P < 0.05$).

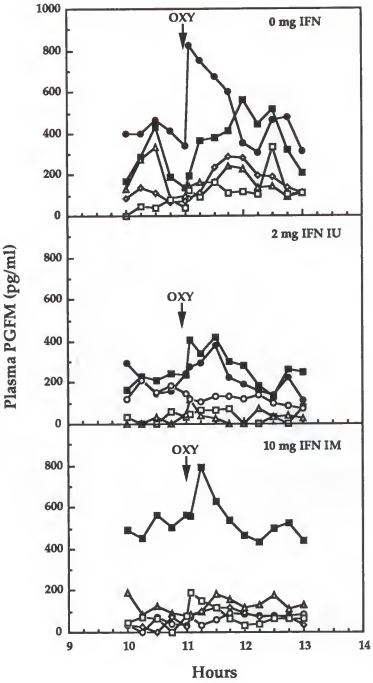


Fig. 7-3. Individual cow profiles of plasma PGFM on Day 17 for cows receiving a control BSA solution, 2 mg rBoIFN in utero or 10 mg rBoIFN intramuscularly. Cows were challenged at 1100 h with an intravenous bolus of 100 U oxytocin (OXY).

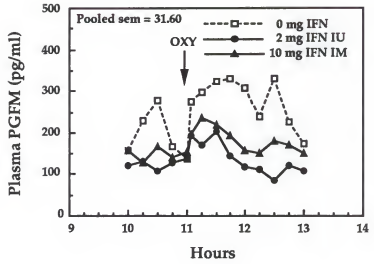


Fig. 7-4. Effect of intrauterine (IU) and intramuscular (IM) administration of rBoIFN on PGFM secretion before and after intravenous administration of 100 U of oxytocin (OXY). Due to heterogeneity of variance among treatments, PGFM data were transformed by square roots prior to analysis. Based on analysis of variance and heterogeneity of regression analysis, PGFM concentrations were similar among treatment groups before the oxytocin challenge (1000-1100 h samples). Oxytocin induced an overall increase in PGFM concentrations ($P < 0.001$). Regression analysis on transformed values from 0-120 min post-oxytocin injection indicated that this increase was lower ($P < 0.05$) for cows treated with rBoIFN compared to control cows, but did not differ between the two rBoIFN-treated groups.

Discussion

In agreement with previous results (Chapter 5), intramuscular and intrauterine administration of rBoIFN extended the interestrous interval and CL lifespan. Results suggest that luteal maintenance involved changes in uterine release of $PGF_{2\alpha}$ as oxytocin-induced PGFM was reduced in rBoIFN-treated cows. This is similar to the situation in early pregnancy (Day 17-19) when endometrial release of $PGF_{2\alpha}$ induced by estradiol (Rico et al., 1981; Thatcher et al., 1984b) and oxytocin (Lafrance and Goff, 1985; Putney et al., 1989b) is attenuated. Infusion of conceptus interferons into the uterus also attenuates uterine release of $PGF_{2\alpha}$. Intrauterine infusion of bCSP from Day 15.5 to Day 21 decreased concentrations of PGFM after an estradiol injection on Day 18 of the cycle (Knickerboker et al., 1986a). In the current study concentrations of PGFM before oxytocin challenge on Day 17 of the estrous cycle were similar for rBoIFN-treated and control cows. Similarly, pregnant and cyclic cows at Days 17-19 post estrus have similar concentrations of PGFM prior to an estradiol (Rico et al., 1981) or oxytocin (Lafrance and Goff, 1985; Putney et al., 1989b) challenge. In-vitro, bTP-1 (Helmer et al., 1989a, 1989b) and other interferons (Salamenson et al., 1988, 1989; Chapter 6) also reduce endometrial release of $PGF_{2\alpha}$.

The follicular population around Day 15 of the cycle plays an important role in luteolysis (Fogwell et al., 1985; Hughes et al., 1987). Since interferons can reduce circulating concentrations of estradiol in women (Kauppila et al., 1982), rBoIFN may delay luteolysis by suppressing production of estrogen from follicles that initiate luteolysis. In the present study, an intensive

survey of follicular growth was not carried out but, nonetheless, no effect of rBoIFN on follicular characteristics or estradiol concentrations were detected.

Interestingly, one cow in the rBoIFN-IM treated group had high post-oxytocin concentrations of PGFM, but still had an extended interestrous interval, possibly because of some other mechanism not involving inhibition of PGFM. In particular, bTP-1 (Helmer et al., 1989a), as well as rBoIFN (3 out of 4 experiments in Chapter 6), increased endometrial secretion of PGE_2 in-vitro. In cattle, intrauterine infusion of PGE_2 and estradiol from Days 13 to 21 extended luteal lifespan compared to cows receiving vehicle, estradiol or PGE_2 (Reynolds et al., 1983). These results suggest that PGE_2 could play a role in the extension of the luteal lifespan.

In the present study, rBoIFN induced an acute pyrogenic effect, with the peak of hyperthermia at 6 h after administration. Similar to previous findings (Newton et al., 1989a), this effect was not detected after subsequent rBoIFN injections. The intramuscular injection of rBoIFN was more pyrogenic than the intrauterine infusion, probably due to dose differences. No pyrogenic effect was detected in Chapter 5 since rectal temperatures were not taken on the first day of injection. At Day 14, a significant decline in serum concentrations of progesterone coincided with the peak of hyperthermia in rBoIFN-IM treated cows. Other depressions in concentrations of progesterone caused by rBoIFN-IM were detected on Days 15-17, though of lower magnitude than on Day 14 and not temporally associated with hyperthermia. From the present study and the literature (Kauppila et al., 1982; Orava et al., 1985, 1986; Orava, 1989), it is clear that IFN molecules can depress circulating concentrations of certain steroids.

The potential use of rBoIFN as an antiluteolytic agent to increase fertility in cattle is appealing. However, the pyrogenic effects of rBoIFN as

well as the depression in progesterone concentrations could be detrimental to conceptus survival and diminishing possible beneficial effects of rBoIFN around Day 17 post breeding. Conceptuses recovered from heat-stressed cows between Days 8-16 of pregnancy are smaller than those from control cows (Biggers et al., 1987). In addition, Day 17 conceptuses which were heat stressed in vitro secreted less bTP-1 than thermoneutral conceptuses (Putney et al., 1988b). Recent studies, however, indicated that pregnancy rate and number of live lambs born increased with twice daily intramuscular injections of 2 mg rBoIFN from Days 11 to 18 after mating (Schalue-Francis et al., 1989; Nephew et al., 1990). Perhaps side effects of rBoIFN are different in sheep than cattle or the side effects in sheep are not sufficient to override positive effects of rBoIFN on fertility.

CHAPTER 8
GENERAL DISCUSSION AND CONCLUSION

In cattle, establishment of gestation requires continual secretion of progesterone to allow development of the histotrophic uterine milieu essential for conceptus survival. Extension of the CL lifespan beyond the normal length of the estrous cycle is ensured during pregnancy because the conceptus signals its presence to the maternal system by reducing uterine $PGF_{2\alpha}$ secretion to prevent the rapid regression of the CL. The biochemical message blocking luteolysis by the bovine conceptus has been identified as a complex of proteins designated bTP-1 (Helmer et al., 1987, 1989a, 1989b). Though in-situ hybridization studies using oTP-1 cDNA probes detected bTP-1 mRNAs in bovine trophectoderm as early as Day 12 of gestation (Farin et al., 1989), large quantities of bTP-1 are not secreted until between Days 17-22 of gestation (Bartol et al., 1985a; Godkin et al., 1988c). Inadequate secretion of bTP-1 may allow luteolysis which and could account for a significant part of the 30% embryonic loss occurs before Days 30-35 of gestation in cattle (Ayalon, 1978). Recent evidence supporting this concept is from studies involving heat stressed in cattle. Conceptuses recovered from severely heat-stressed cows between Days 8-16 were smaller than those from control cows (Biggers et al., 1987). Geisert et al. (1988a) reported that Days 15-17 bovine conceptuses of 17 mm secrete only small amounts of bTP-1 compared to elongating conceptuses. In another study, Putney et al. (1988b) demonstrated that Day 17 conceptuses exposed to elevated temperature in vitro secreted less bTP-1 than

those cultured at 39°C. Geisert et al. (1988b) did not detect a qualitative decrease in production of bTP-1 in-vitro by conceptuses exposed to in-vivo heat-stress when evaluating fluorographs following 2D SDS-PAGE. It is, therefore, difficult to estimate total production of bTP-1. A retarded heat-stressed conceptus may not secrete sufficient bTP-1 to extend CL lifespan. Moreover, other situations, such as viral and bacterial infection, may also affect the secretion of bTP-1.

It seems reasonable to consider that administration of bTP-1 around the critical period of pregnancy recognition could enhance survival of bTP-1 deficient conceptuses by prolonging luteal lifespan. The hypothesis that maintaining CL function can increase fertility, is supported by several lines of evidence. Thatcher et al. (1987) administered hCG at Day 15 of gestation to extend CL function and improved fertility rates by 1-7%. A similar increase in pregnancy rate (11.5%) was obtained with one injection of 10 μg gonadotropin releasing hormone analogue between Days 11 and 13 post breeding (Macmillan et al., 1986). Pregnancy rate was also higher (17%) with cotransfer of a cryopreserved Day 8 bovine embryo and two Day 14 bovine trophoblastic vesicles (Heyman et al., 1987), probably due to increased amounts of conceptus tissues secreting bTP-1. Therefore, administration of exogenous bTP-1 may enhance conceptus survival and may prove to be an effective fertility drug.

This hypothesis has been difficult to test because purification of bTP-1 is laborious (Helmer et al., 1989b; Chapter 3) and small yields of bTP-1 are derived from in-vitro culture of bovine conceptuses. In the future, it may be possible to use synthetic or recombinant bTP-1 peptides in place of native bTP-1. Results from Chapter 4 indicate that the C-terminal portion (152-172 a.a.) of bTP-1 plays an important role in antigenicity of the molecule, and use of this peptide should allow alternative methods of producing bTP-1 antibodies

without using purified bTP-1. However, none of the three tested peptides (100-113, 131-140, 152-172 a.a.) had antiviral activity characteristic of bioactive bTP-1 (Godkin et al., 1988b; Chapter 3). Results of previous studies indicated that three distinct domains were important for active configuration of HuIFN and its binding to cellular receptors (Fish et al., 1989). Thus, future research to develop biologically active bTP-1 peptides should involve synthesis of peptides possessing both antiviral and antiluteolytic activities Alternatively, nine bTP-1 isomers could be produced through recombinant DNA techniques and tested for antiviral and antiluteolytic properties.

Because recombinant bTP-1 is not available in significant amount; recombinant bovine alpha interferon may be an alternative protein for regulating CL function and fertility in cattle. The bTP-1 complex shares 80%, 72% and 50% amino acid sequence homology with oTP-1, bovine interferon α class II and bovine interferon α class I (rBoIFN), respectively (Imakawa et al., 1989). Immunological cross-reactivity among bTP-1, oTP-1 and rBoIFN were also demonstrated (Chapter 3). Unfortunately, neither oTP-1 nor bovine interferon α class II are currently available in large amounts and can not be used as substitutes for bTP-1. However, large quantities of recombinant bovine interferon-α_I are available which led us to test whether the biological antiluteolytic properties of bTP-1 are shared with rBoIFN.

Intrauterine infusion of rBoIFN prolonged interestrous intervals and CL lifespan in cattle. Unfortunately, intrauterine infusion of rBoIFN during the early stages of pregnancy is not feasible as it may be detrimental to the developing conceptus. Moreover, the more practical route of intramuscular administration of rBoIFN also extended the interestrous interval and the CL lifespan (Chapters 5 and 7).

The bTP-1 complex possesses antiviral activity (Godkin et al., 1988b; Chapter 3), decreases incorporation of radiolabelled amino acids into secreted endometrial proteins, reduces $PGF_{2\alpha}$ secretion by endometrium (Helmer et al., 1989a; Chapter 6) and extends luteal lifespan when infused in utero by suppressing uterine release of $PGF_{2\alpha}$ (Helmer et al., 1989b). The bTP-1 complex is recognized as an antiluteolytic-anti-PGF protein which activates an inhibitor of enzymes necessary for synthesis of $PGF_{2\alpha}$. In addition, bTP-1 increased (Helmer et al., 1989a) or decreased (Chapter 6) secretion of PGE_2 in-vitro by bovine endometrial explants. Even though rBoIFN extends luteal lifespan (Chapters 5 and 7) and decreases $PGF_{2\alpha}$ release in response to oxytocin, it is unclear whether rBoIFN inhibits endometrial synthesis of $PGF_{2\alpha}$ through the same mechanisms as bTP-1. Recombinant BoIFN inhibited secretion of PGF by bovine endometrial explants in only one of four experiments and PGE_2 secretion was increased in three of the four experiments.

Ovine TP-1 prevents regression of ovine CL through a similar mechanism to bTP-1. Reciprocal interspecies transfer of trophoblastic vesicles into the uteri of cyclic recipients extends CL function (Martal et al., 1984) and oTP-1 depresses secretion of PGF by bovine endometrial explants (Chapter 6). The oTP-1 complex also extends interestrous interval by suppressing endometrial secretion of $PGF_{2\alpha}$ (Godkin et al., 1984b; Vallet et al., 1988). However, oTP-1 does not escape from the uterine lumen and appears to act directly upon the endometrium as a paracrine hormone (Godkin et al., 1984a). Only negligible amounts of radioactivity reached the ovaries when radiolabelled oTP-1 was infused in utero in Day 12 cyclic ewes thereby suggesting that oTP-1 does not leave the uterine lumen through either utero-

ovarian vessels or the lymphatic pathway. Bovine TP-1 is also hypothesized to be a paracrine hormone acting on endometrial cells.

There are at least five mechanisms whereby rBoIFN may prolong luteal lifespan. First, rBoIFN may act directly on the uterine endometrium like bTP-1. For this to occur, rBoIFN must be sequestered in the uterine lumen following intramuscular injection. In support of this idea, rBoIFN decreased oxytocin-induced endometrial secretion of PGFM in-vivo (Chapter 7), reduced secretion of PGF by bovine endometrial explants in-vitro in one experiment (Chapter 6) and reduced secretion of PGF by ovine endometrial cells in-vitro (Salamonsen et al., 1987, 1989). Second, rBoIFN (administered either IM or IU) may decrease oxytocin-induced PGFM release through systemic mechanisms which differ from the local effect of bTP-1. Recombinant BoIFN does not share 100% homology with bTP-1 and inherent differences in the protein may allow it to enter into the maternal circulation following intrauterine administration. This possibility is supported by the fact that both intramuscular and intrauterine administration of rBoIFN resulted in systemic effects (rBoIFN-IM and IU increased body temperature and rBoIFN-IM decreased progesterone; Chapter 7). Third, rBoIFN may affect development of follicles critical for uterine $PGF_{2\alpha}$ release. However, the current results of this study did not detect any effect of rBoIFN on follicular characteristics (Chapter 7). Fourth, rBoIFN may act directly on the CL. Godkin et al. (1984a) demonstrated that oTP-1 does not stimulate progesterone production by dispersed luteal cells from Day 12 cycling ewes, but does bind to luteal receptors that are independent of LH and hCG receptors. Human IFN and rBoIFN also bind to the same ovine endometrial receptors as oTP-1 (Stewart et al., 1987; Hansen et al., 1989), and it is likely, therefore, that rBoIFN binds to bovine luteal receptors for interferons.

Though completely undefined experimentally, binding of interferons to their luteal receptors may decrease or increase the number of cellular receptors available for other molecules by altering either their synthesis or degradation (Faltynek et al., 1984; Taylor-Papadimitriou and Rozengurt, 1985). Recombinant BoIFN may also indirectly affect the CL by increasing endometrial secretion of PGE_2. In three of four in-vitro experiments, rBoIFN increased endometrial secretion of PGE_2 (Chapter 6). In cattle, Gimenez and Henricks (1983) extended CL lifespan with intrauterine infusion of PGE_2 from Days 9-12 of the estrous cycle. In another study, intrauterine infusion of PGE_2 and estradiol from Days 13 to 21 also prolonged luteal lifespan compared to cows receiving vehicle alone, estradiol or PGE_2 (Reynolds et al., 1983). Prostaglandin E_2, acting synergistically with estradiol, may directly or indirectly influence the CL. In-vitro studies have indicated that PGE_2 has a luteotrophic effect on bovine luteal cells by increasing the synthesis of progesterone (Speroff and Ramwell, 1970; Marsh, 1970). In mid-cycle, large bovine luteal cells, which constitute approximately 40% of the luteal tissue, secrete most of the circulating progesterone (O'Shea et al., 1989). These large luteal cells are very responsive to stimulation by PGE_2 (Fitz et al., 1984a, 1984b). Prostaglandin E_2 is also a potent vasodilator and can increase utero-ovarian blood flow (Thatcher et al., 1984b). Around Day 15 of pregnancy, Reynolds (1986) suggested that an increase in blood flow coincides with an increase in circulating progesterone to inhibit the luteolytic effect of endometrial $PGF_{2\alpha}$.

Intramuscular administration of rBoIFN induces a severe pyrogenic effect which is associated with an acute decrease in concentrations of circulating progesterone. Only a slight hyperthermia is induced by intrauterine administration of rBoIFN. Unfortunately, intrauterine infusion

of rBoIFN during the early stages of pregnancy may be detrimental to the developing conceptus. Intramuscular administration, though not directly injurious to the conceptus, could be detrimental due to its induction of hyperthermia and lowered concentrations of progesterone. Additional investigations of routes of administration, doses and antiluteolytic mechanism(s) of action are required to assess the value of using rBoIFN as a substitute for bTP-1.

The natural selection of an interferon for the pregnancy recognition signal in cattle and sheep is intriguing. In addition to their antiluteolytic effects, bTP-1 and oTP-1 also display other activities characteristic of alpha IFNs, such as immunosuppressive, antiproliferative and antiviral activities (Godkin et al., 1988b; Pontzer et al., 1988; Newton et al., 1989b; Niwano et al., 1989; Roberts et al., 1989; Chapter 3). It is possible therefore, that bTP-1 effects in utero are for additional purposes. Bovine TP-1 may protect the conceptus from pathogenic microorganisms and control cellular growth in the conceptus after embryonic loss of the zona pellucida. Differences in the mitotic index between polar and mural trophectoderm cells is well documented in mice (Barlow et al., 1972; Copp, 1978, 1979) led to the hypothesis that different centers of proliferation and differentiation exist within the developing blastocysts (Dickson, 1966; Denker, 1983). The integration of antiproliferative agents of conceptus origin into such a model may prove instructive. This will be particularly true if the early conceptus has bTP-1 receptors on its trophectoderm for bTP-1.

In conclusion, the bTP-1 complex contains at least 9 isomers, which were purified using ultrafiltration units and HPLC anion-gel filtration chromatography. Bovine TP-1 has antiviral activity and immunological

cross-reactivity with rBoIFN. In-vivo, administration of rBoIFN causes extension of luteal lifespan that involves decreased responsiveness of the endometrium to oxytocin-induced release of $PGF_{2\alpha}$. However, pyrogenic effects and inhibiting effects of progesterone secretion must be studied further before rBoIFN can be used for enhancing fertility in cattle.

APPENDIX
EFFECT OF rBoIFN ON IN-VITRO DEVELOPMENT OF DAY 8 BOVINE EMBRYOS

Materials and methods

Fifty-one morulae and blastocysts were collected nonsurgically from superovulated cows on three different occasions. The embryos were cultured under 5:95 (v/v) CO_2:air (38.5 °C) with 0, 1, 10 or 100 μg rBoIFN dissolved in 1 ml of Hams F-10 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml L-glutamine (Sigma Chemical Co, St Louis, MO). Total protein was adjusted to 440 μg/ml with BSA (fraction V, Sigma). At the beginning of each experiment, all embryos were classified as fair, good or excellent. After 24 h of culture, all embryos were reclassified and scored as either degenerate (1), unaffected (2) or advanced in development (3).

Data on development rate were analyzed by a least squares analysis using general linear and CATMOD procedure of SAS (1985). The model considered effects of experiment, cow, experiment x cow, treatment, experiment x treatment, cow x treatment and experiment x cow x treatment. A simplified model considered treatment and scoring effects. Differences in treatment means were evaluated by orthogonal contrasts (0 vs 1, 10 and 100 μg; 1 vs 10 and 100 μg; 10 vs 100 μg).

Results

Individual scoring of embryonic development is presented in Table A-1. Mean scores for embryos receiving 0, 1, 10 or 100 μg/ml rBoIFN were not

affected by treatment and averaged 1.91 (n=11), 2.00 (n=14), 2.20 (n=15) and 2.55 (n=11), respectively.

Table A-1. Development rate of Day 8 bovine embryos after 24 h of in-vitro culture in medium containing various levels of rBoIFN. At the end of the culture period, all embryos were morphologically classified and scored as either degenerate (1), unaffected (2) or advanced in development (3).

	Concentrations of rBoIFN (μg/ml)			
Scoring	0	1	10	100
1	5*	6	5	2
2	2	2	2	1
3	4	6	8	8

* Number of embryos

Conclusion

Recombinant BoIFN did not directly affect in vitro development of Day 8 bovine embryos.

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BIOGRAPHICAL SKETCH

Claire Plante was born in St-Valère, Québec, in 1957. In the fall of 1978, she enrolled at the Faculté de Médecine Vétérinaire in St-Hyacinthe, Québec, and graduated in spring 1982. She practiced 3 months at the Clinique Ambulatoire at the Faculté de Médecine Vétérinaire before entering a Master of Science program at the Centre de Recherche en Reproduction Animal, St-Hyacinthe. She obtained her Master of Science degree in October 1984. She pursued further clinical training in Theriogenology at Iowa State University from July 1984 to July 1986. During that period, besides learning English and improving her clinical skills, she had the opportunity to meet her future husband. In September 1986, she began her doctoral studies with Drs. Peter J. Hansen and William W. Thatcher at the University of Florida in Gainesville. She obtained her American board certification in Theriogenology in 1987. Since September 1989, she is working with Dr. Richard B. Miller at the Ontario Veterinary College, in Guelph, under a fellowship granted by the Medical Research Council of Canada.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Peter J. Hansen, Chair
Associate Professor of
Dairy Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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May 1990

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